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<p>(54) Title: METHODS OF MODULATING TUMOR NECROSIS FACTOR <math>\alpha</math>-INDUCED EXPRESSION OF CELL ADHESION MOLECULES</p> <p><b>TNF<math>\alpha</math> :</b> - + + + + + +</p> <table border="0"> <tr> <td><b>Antisense Oligo:</b></td> <td><b>None</b></td> <td><b>C-raf</b></td> <td><b>Control</b></td> <td></td> </tr> <tr> <td><b>Time:</b></td> <td>0 5 15</td> <td>5 15</td> <td>5 15</td> <td>Min.</td> </tr> </table> <p><b>ERK Activity</b> <b>phospho-ELK</b></p> <p><b>JNK Activity</b> <b>phospho-CJUN</b></p> <p><b>p38 Activity</b> <b>phospho-ATF-2</b></p> <p><b>(57) Abstract</b></p> <p>Methods are provided for inhibiting the expression of cell adhesion molecules using inhibitors of signaling molecules involved in human TNF-<math>\alpha</math> signaling. These inhibitors include monoclonal antibodies, peptide fragments, small molecule inhibitors, and, preferably, antisense oligonucleotides. Methods for treatment of diseases, particularly inflammatory and immune diseases, associated with overexpression of cell adhesion molecules are provided.</p>				<b>Antisense Oligo:</b>	<b>None</b>	<b>C-raf</b>	<b>Control</b>		<b>Time:</b>	0 5 15	5 15	5 15	Min.
<b>Antisense Oligo:</b>	<b>None</b>	<b>C-raf</b>	<b>Control</b>										
<b>Time:</b>	0 5 15	5 15	5 15	Min.									

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**METHODS OF MODULATING TUMOR NECROSIS FACTOR  $\alpha$ -INDUCED  
EXPRESSION OF CELL ADHESION MOLECULES**

**FIELD OF THE INVENTION**

5       The present invention relates to the modulation of expression of cell adhesion molecules. In particular, herein are provided methods of inhibiting cell adhesion molecule gene expression through specific inhibitors involved in TNF- $\alpha$  signaling. Methods are also provided for  
10      treating inflammatory and immune diseases associated with altered expression of cell adhesion molecules.

**BACKGROUND OF THE INVENTION**

Cytokines represent a diverse group of regulatory proteins with numerous biological functions including cell differentiation, cell growth, and cytotoxicity. Inflammatory cytokines such as Tumor Necrosis Factor alpha and (TNF- $\alpha$ ) and IL-1 (interleukin-1) have been shown to play pivotal roles in immune and inflammatory responses (McIntyre, T.M., et al., Thromb. Haemos. 1997, 78, 302-305). One of the most important effector functions of these cytokines is their ability to induce profound changes in the vascular endothelium (Introna, M. and Mantovani, A., Art. Thromb. and Vasc. Biol. 1997, 17, 423-428). Central to the process of inflammation is the induction of cell adhesion molecules on the endothelial cell surface, contributing significantly to the adherence and recruitment of circulating leukocytes to inflammatory sites. Upon exposure to TNF- $\alpha$  or IL-1, which are produced in response to injury or infection, cytokine receptors on endothelial cells activate a variety of intracellular signaling molecules. These signaling events result in the activation of specific transcription factors such as NF-kB and upregulate the expression of E-selectin, ICAM-1, VCAM-1, and other cell adhesion molecules (McIntyre, T.M., et al., Thromb. Haemos. 997, 78, 302-305; Introna, M. and Mantovani, A., Art. Thromb. and Vasc. Biol.

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1997, 17, 423-428; Mantovani, A., et al., Thromb. Haemos. 1997, 78, 406-414). E-selectin has been shown to mediate the initial attachment and rolling of leukocytes along the vessel wall, whereas ICAM-1 and VCAM-1 are involved in the firm adhesion of leukocytes to the vessel wall and their transmigration through the vessel wall. E-selectin is rapidly and transiently induced by cytokines with peak expression occurring approximately 4-6 hours after exposure and returning to basal levels approximately 24 hours post exposure. In contrast, induction of ICAM-1 and VCAM-1 by cytokines is slower and persists for 24 hours or longer (Mantovani, A., et al., Thromb. Haemos. 1997, 78, 406-414; Dunon, D., et al., Curr. Opin. Cell Bio., 1996, 8, 714-723; Bischoff, J., Cell Adhes. and Angiog., 1997, 99, 373-376).

15        Responses to TNF- $\alpha$  are mediated through interactions with two distinct membrane receptors, termed TNFRI (TNF- $\alpha$  receptor I) and TNFRII (TNF- $\alpha$  receptor II). Two distinct families of adaptor proteins associated with TNF- $\alpha$  receptors have been identified. The death domain-containing proteins (e.g., TRADD) appear to couple the receptors to programmed cell death (Fiers, W., et al., J. Inflam., 1996, 47, 67-75; Wallach, D., et al., FEBS Lett., 1997, 410, 96-106; Hsu, H., et al., Cell, 1996, 84, 299-308), whereas the TRAF (TNF receptor associated factor) domain-containing proteins link the receptors to activation of specific transcription factors (Hsu, H., et al., Cell, 1996, 84, 299-308; Baeuerle, P.A., Curr. Biol., 1998, 8, R19-R22). Among the six members of the TRAF family that have been identified so far, TRAF2 has been reported to be important for TNF- $\alpha$ -mediated activation of JNK (c-Jun N-terminal kinase), as well as two major transcription factors, NF- $\kappa$ B (nuclear factor- $\kappa$ B) and AP-1 (activator protein 1) (Hsu, H., et al., Cell, 1996, 84, 299-308; Baeuerle, P.A., Curr. Biol., 1998, 8, R19-R22; Natoli, G., et al., J. Biol. Chem., 1997, 272, 26079-26082; Liu, Z.G.,

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et al., Cell, 1996, 87, 565-576; Song, H.Y., et al., Proc. Natl. Acad. Sci. USA, 1997, 94, 9792-9796). Both transcription factors play pivotal roles in the regulation of multiple genes including those involved in immune and inflammatory responses. AP-1 is activated by various MAPKs (mitogen-activated protein kinase) including ERK (extracellular-signal-regulated kinase), JNK and p38 MAPK (Fiers, W., et al., J. Inflam., 1996, 47, 67-75; Eder, J., TIPS, 1997, 18, 319-322). NF- $\kappa$ B is constitutively present in the cytosol of endothelial cells and kept inactive by association with inhibitory I $\kappa$ B family proteins. Upon exposure to TNF- $\alpha$ , IKK (I $\kappa$ B kinase) phosphorylates I $\kappa$ B and initiates its ubiquitination and subsequent degradation. The released NF- $\kappa$ B translocates to the nucleus and participates in transcriptional activation (Collins, T., et al., FASEB J., 1995, 9, 899-909; Stancovski, I., and Baltimore, D., Cell, 1997, 91, 299-302).

Other signaling molecules, including MEKK1, pp90rsk (ribosomal S6 protein kinase), ras, and raf, have been implicated in the activation of NF- $\kappa$ B (Schulze-Osthoff, K., et al., Immunobiol., 1997, 198, 35-49). ras family members (Ha-ras, Ki-ras, N-ras) are GTP-binding proteins that act as major mediators in the regulation of cell proliferation and differentiation in response to a variety of extracellular stimuli including TNF- $\alpha$  (Bos, J.L., Biochem. Biophys. Acta, 1997, 1333, M19-M31). ras proteins have been shown to activate both the raf/MEK/ERK pathway as well as MEKK/JNKK/JNK pathway (Bos, J.L., Biochem. Biophys. Acta, 1997, 1333, M19-M31; Marais, R., and Marshall, C.J., Cancer Surveys, 1996, 27, 101-125; Adler, V., et al., J. Biol. Chem., 1996, 271, 23304-23309; Faris, M., et al., J. Biol. Chem., 1996, 271, 27366-27373; Terada, K., et al., J. Biol. Chem., 1997, 272, 4544-4548). raf family members (A-, B-, c-raf) are serine/threonine protein kinases that transmit signals from cell surface receptors to a variety

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of intracellular effectors including the MAPK pathways (Marais, R., and Marshall, C.J., Cancer Surveys, 1996, 27, 101-125; Daum, G., et al., TIBS, 1994, 19, 474-480). Besides *ras*, a variety of protein kinases including Src family kinases and PKC (protein kinase C) can potentiate *raf* activity (Marais, R., et al., J. Biol. Chem., 1997, 272, 4378-4383; Ueffing, M., et al., Oncogene, 1997, 15, 2921-2927). The major downstream effectors of *raf* are MEK/MKK1 (MAP kinase kinase 1) and MEK/MKK2 (MAP kinase kinase 2) which in turn phosphorylate and activate ERK1/2, and ultimately activate specific transcription factors (Marais, R., and Marshall, C.J., Cancer Surveys, 1996, 27, 101-125; Daum, G., et al., TIBS, 1994, 19, 474-480). Both *ras* and *raf* had been suggested to participate in the activation of NF- $\kappa$ B transcription factors (Schulze-Osthoff, K., et al., Immunobiol., 1997, 198, 35-49; Folgueira, L., et al., J. Virol., 1996, 70, 2332-2338; Koong, A.C., et al., Cancer Res., 1994, 54, 5273-5279; Bertrand, F., et al., J. Biol. Chem., 1995, 270, 24435-24441; Kanno, T., and Siebenlist, U., J. Immunol., 1996, 157, 5277-5283).

In many human diseases with an inflammatory component, the normal, homeostatic mechanisms which attenuate the inflammatory responses are defective, resulting in damage and destruction of normal tissue. For example, VCAM-1 may play a role in the metastasis of melanoma, and possibly other cancers. In addition, data have demonstrated that ICAM-1 is the cellular receptor for the major serotype of rhinovirus, which account for greater than 50% of common colds. (Staunton, et al., Cell, 1989, 56, 849-853; Greve et al., Cell, 1989, 56, 839-847).

Expression of ICAM-1 has also been associated with a variety of inflammatory skin disorders such as allergic contact dermatitis, fixed drug eruption, lichen planus, and psoriasis (Ho, et al., J. Am. Acad. Dermatol., 1990, 22, 64-68; Griffiths and Nickoloff, Am. J. Pathology, 1989,

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135, 1045-1053; Lisby, et al., Br. J. Dermatol., 1989, 120,  
479-484; Shiohara, et al., Arch. Dermatol., 1989, 125,  
1371-1376). In addition, ICAM-1 expression has been  
detected in the synovium of patients with rheumatoid  
5 arthritis (Hale, et al., Arth. Rheum., 1989, 32, 22-30),  
pancreatic B-cells in diabetes (Campbell, et al., Proc.  
Natl. Acad. Sci. U.S.A., 1989, 86, 4282-4286), thyroid  
follicular cells in patients with Graves' disease (Weetman,  
et al., J. Endocrinol., 1989, 122, 185-191), and with renal  
10 and liver allograft rejection (Faull and Russ,  
Transplantation, 1989, 48, 226-230; Adams, et al., Lancet,  
1989, 2, 1122-1125).

Inhibitors of ICAM-1, VCAM-1 and ELAM-1 expression  
would provide a novel therapeutic class of anti-  
15 inflammatory agents with activity towards a variety of  
inflammatory diseases or diseases with an inflammatory  
component such as asthma, rheumatoid arthritis, allograft  
rejections, inflammatory bowel disease, various  
dermatological conditions, and psoriasis. In addition,  
20 inhibitors of ICAM-1, VCAM-1, and ELAM-1 may also be  
effective in the treatment of colds due to rhinovirus  
infection, AIDS, Kaposi's sarcoma and some cancers and  
their metastasis. The use of neutralizing monoclonal  
antibodies against ICAM-1 in animal models provide evidence  
25 that such inhibitors if identified would have therapeutic  
benefit for asthma (Wegner, et al., Science, 1990, 247,  
456-459), renal allografts (Cosimi, et al., J. Immunol.,  
1990, 144, 4604-4612), and cardiac allografts (Isobe, et  
al., Science, 1992, 255, 1125-1127). The use of a soluble  
30 form of ICAM-1 molecule was also effective in preventing  
rhinovirus infection of cells in culture (Marlin, et al.,  
Nature, 1990, 344, 70-72).

Current agents which affect intercellular adhesion  
molecules include synthetic peptides, monoclonal  
35 antibodies, soluble forms of the adhesion molecules, and

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antisense oligonucleotides. Antisense oligonucleotides to cell adhesion molecules are disclosed in US Patent Nos. 5,514,788 and 5,591,623, herein incorporated by reference. These have been directed against a single cell adhesion molecule. Additional agents are desired. Furthermore, a broader approach, targeting several adhesion molecules with a single agent may have several advantages, including economies of scale, broad spectrum utility, etc. Thus, an approach to target a molecule in the TNF- $\alpha$  signaling pathway may be a useful therapeutic treatment, providing a means to regulate multiple cell adhesion molecules with a single agent.

Inhibitors of molecules in TNF- $\alpha$  mediated signaling have been used to study the signal transduction pathways and suggest utility in the design of pharmacological agents. Inhibitors that have been used include DMSO (Essani, N.A., et al., Shock, 1997, 7, 90-96) against NF- $\kappa$ B, protein tyrosine kinase inhibitors (Adamson, P., et al., Cell Adhes. Commun., 1996, 3, 511-525; Pai, R., et al., J. Immunol., 1996, 156, 2571-2579), protein tyrosine kinase C inhibitors (Ballestas, M.E. and Benveniste, E.N., Glia, 1995, 14, 267-278), ubiquitin ligase inhibitors (Yaron, A., et al., EMBO J., 1997, 16, 6486-6494), and phospholipase A2 inhibitors (Thommesen, L., et al., J. Immunol., 1998, 161, 3421-3430). In addition, drugs that elevate cyclic AMP have been found to inhibit ELAM-1 and VCAM-1 (Poher, J.S., et al., J. Immunol., 1993, 150, 5114-5123).

Antisense oligonucleotides to c-raf, Ha-ras and JNK2 are known, but have not previously been shown to inhibit cell adhesion molecule expression. The relationship between these TNF- $\alpha$  signaling molecules and cell adhesion molecule expression has not been fully delineated. c-raf antisense oligonucleotides are disclosed in US Patent Nos. 5,563,255 and 5,656,612, herein incorporated by reference. Ha-ras antisense oligonucleotide are disclosed in US Patent

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Nos. 5,576,208 and 5,582,986, herein incorporated by reference. JNK2 antisense oligonucleotides are disclosed by Bost, F., et al. (J. Biol. Chem. 1997, 272, 33422-33429). Inhibitors of the TNF- $\alpha$  signaling molecules, *c-raf*, Ha-ras and JNK2 have not been used to modulate expression of cell adhesion molecules and represent a novel approach.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a Western blot showing a time-course of the effects of *c-raf* antisense oligonucleotides on *c-raf* and *a-raf* protein levels.

FIG. 2 is a Western blot showing a time-course of the effects of Ha-ras antisense oligonucleotides on Ha-ras and Ki-ras protein levels.

FIG. 3 is a Western blot showing the effects of *c-raf* antisense oligonucleotides on TNF- $\alpha$  mediated ERK, JNK and p38 kinase activities. Phospho-substrate-specific antibodies were used to analyze kinase activities.

FIG. 4 is Northern blot showing the effects of JNK1 and JNK2 antisense oligonucleotides on TNF- $\alpha$  mediated JNK1 and JNK2 mRNA expression.

FIG. 5 is a Western blot showing the effects of JNK1 and JNK2 antisense oligonucleotides on TNF- $\alpha$  mediated JNK1 and JNK2 kinase activity. Phospho-substrate-specific antibodies were used to analyze kinase activities.

#### BRIEF DESCRIPTION OF THE INVENTION

The present invention describes a method of modulating cell adhesion molecule expression within a cell comprising treating said cell with a specific inhibitor of one of the Tumor Necrosis Factor alpha (TNF- $\alpha$ ) signaling molecules, Ha-ras, *c-raf* or JNK2. In one embodiment, the specific inhibitor is an antisense oligonucleotide capable of hybridizing to Ha-ras, *c-raf* or JNK2. Also provided are methods of treating an inflammatory or immune disease or

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condition associated with altered expression of a cell adhesion molecule comprising administering a specific inhibitor of one of the TNF- $\alpha$  signaling molecules, Ha-ras, c-raf or JNK2.

## 5 DETAILED DESCRIPTION OF THE INVENTION

The present invention employs specific inhibitors of Ha-ras, c-raf and JNK2, members of the TNF- $\alpha$  signaling pathway, to modulate cell adhesion molecule expression. These inhibitors can include monoclonal antibodies, peptide 10 fragments, small molecule inhibitors and antisense compounds. In a preferred embodiment, antisense compounds, particularly oligonucleotides, are used to modulate the function of nucleic acid molecules encoding Ha-ras, c-raf or JNK2, modulating the amount of protein produced and 15 ultimately modulating the expression of cell adhesion molecules. This is accomplished by providing oligonucleotides which specifically hybridize with nucleic acids, preferably mRNA, encoding Ha-ras, c-raf or JNK2.

This relationship between an antisense compound such 20 as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a multistep process. The process usually begins with 25 identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the 30 targets are nucleic acids encoding Ha-ras, c-raf or JNK2; in other words, a gene encoding Ha-ras, c-raf or JNK2, or mRNA expressed from the Ha-ras, c-raf or JNK2 gene. mRNA which encodes Ha-ras, c-raf or JNK2 is presently the preferred target. The targeting process also includes

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determination of a site or sites within the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular

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set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding Ha-ras, c-raf or JNK2, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, *i.e.*, 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region," "AUG region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation initiation codon. This region is a preferred target region. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation termination codon. This region is a preferred target region. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or

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corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to 5 include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as 10 "introns," which are excised from a pre-mRNA transcript to yield one or more mature mRNA. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, *i.e.*, exon-exon or intron-exon junctions, may 15 also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also 20 preferred targets. Targeting particular exons in alternatively spliced mRNAs may also be preferred. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

25 Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, *i.e.*, hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

30 "Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary 35 bases which are known to form three hydrogen bonds between

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them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to 10 be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to 15 avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment and, in the case of *in vitro* assays, under conditions in which the 20 assays are conducted.

Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of 25 the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense 30 oligonucleotide hybridization to the RNA.

The overall effect of interference with mRNA function is modulation of expression of *c-raf*, *Ha-ras* or *JNK2* and, in the context of this invention, ultimately modulation of cellular adhesion molecule expression. In the context of 35 this invention "modulation" means either inhibition or

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stimulation; i.e., either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, or reverse transcriptase PCR, as taught 5 in the examples of the instant application or by Western blot or ELISA assay of protein expression, or by an immunoprecipitation assay of protein expression. Effects on cell proliferation or tumor cell growth can also be measured, as taught in the examples of the instant 10 application. Inhibition is presently preferred.

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, and as research reagents and in kits. Since the oligonucleotides of this 15 invention hybridize to nucleic acids encoding Ha-ras, c-raf or JNK2, sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotide with the Ha-ras, c-raf or JNK2 gene or mRNA can routinely be accomplished. Such provision may include enzyme 20 conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of Ha-ras, c-raf or JNK2 may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states in tissue or other samples 25 from patients suspected of having an inflammatory disease such as rheumatoid arthritis. The ability of the oligonucleotides of the present invention to inhibit inflammatory processes may be employed to diagnose such states. A number of assays may be formulated employing the 30 present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells 35 with an oligonucleotide or oligonucleotides means to add

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the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal.

5       The oligonucleotides of this invention may also be used for research purposes. For example, the function of a specific gene product in a signaling pathway may be investigated using specific oligonucleotides. Thus, the specific hybridization exhibited by the oligonucleotides  
10      may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of  
15      ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified  
20      or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

25      The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (*i.e.* from about 8 to about 30 linked  
30      nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further  
35      include a phosphate group covalently linked to the sugar

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portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups 5 covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide 10 structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds 15 useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone 20 and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, 25 for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral 30 phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and 35 those having inverted polarity wherein the adjacent pairs

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of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 10 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); 20 siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide 25 backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 30 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

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In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen et al. (Science, 1991, 254, 1497-1500).

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH<sub>2</sub>-NH-O-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-O-CH<sub>2</sub>- [known as a methylene (methylimino) or MMI backbone], -CH<sub>2</sub>-O-N(CH<sub>3</sub>)-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-N(CH<sub>3</sub>)-CH<sub>2</sub>- and -O-N(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>- [wherein the native phosphodiester backbone is represented as -O-P(=O)-CH<sub>2</sub>-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and

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alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m  
5 are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl,  
10 heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and  
15 other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes  
20 2'-dimethylaminoxyethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar  
30 mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;  
35 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;

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5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531  
5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, 1990, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, those disclosed by Englisch et al. (Angewandte Chemie, International Edition, 1991, 30, 613-722), and those disclosed by Sanghvi, Y.S., Chapter 15, Antisense Research and Applications, 1993, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2-, N-6 and O-6 substituted purines, including 2-

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aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds.,

5 Antisense Research and Applications, 1993, CRC Press, Boca Raton, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 15 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053-1059), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Lett., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-35 hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al.,

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Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantan acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta., 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937).

10 Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 15 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 20 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

25 The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at 30 least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a 35

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substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in 5 cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. This RNase H- 10 mediated cleavage of the RNA target is distinct from the use of ribozymes to cleave nucleic acids. Ribozymes are not comprehended by the present invention.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions 15 are present, normally with a central region flanked by two regions which are chemically equivalent to each other but distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H 20 and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., fluoro- or 2'-O-methoxyethyl- substituted). Chimeric oligonucleotides 25 are not limited to those with modifications on the sugar, but may also include oligonucleosides or oligonucleotides with modified backbones, e.g., with regions of phosphorothioate (P=S) and phosphodiester (P=O) backbone linkages or with regions of MMI and P=S backbone linkages. 30 Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, 35 whereas the 3' portion is modified in such a fashion so as

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to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention 5 contain a 2'-O-methoxyethyl ( $2'-O-CH_2CH_2OCH_3$ ) modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of the oligonucleotide. According to the invention, one, a 10 plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl ( $-O-CH_2CH_2OCH_3$ ) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on 15 any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleotides containing other modifications which enhance antisense efficacy, potency or target affinity are 20 also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. 25 Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to 30 prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta*, 1995, 78, 486-504). It is also well known to use similar techniques and commercially available modified 35 amidites and controlled-pore glass (CPG) products such as

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biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling, VA) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

5       The antisense compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon  
10 administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and  
15 prodrugs of such nucleic acids. "Pharmaceutically acceptable salts" are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired  
20 toxicological effects thereto (see, for example, Berge et al., "Pharmaceutical Salts," J. of Pharma Sci., 1977, 66, 1-19).

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-

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toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

5       The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells  
10 thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO  
15 93/24510 to Gosselin et al., published December 9, 1993.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may  
20 include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the  
25 like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary  
30 delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, 8, 91-192; Muranishi, Critical Reviews in Therapeutic Drug

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Carrier Systems, 1990, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as 5 penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, 10 glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., Critical Reviews in 15 Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1; El-Hariri et al., J. Pharm. Pharmacol., 1992 44, 651-654).

The physiological roles of bile include the 20 facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their 25 synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives.

Complex formulations comprising one or more 30 penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations.

Chelating agents include, but are not limited to, 35 disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate

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and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) [Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., J. Pharm. Pharmacol., 1988, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a

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common receptor. In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering 5 one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other 10 components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other 15 sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn 20 starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage 25 forms are described in U.S. Patents Nos. 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at 30 their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional 35 materials useful in physically formulating various dosage

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forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere  
5 with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to  
10 enhance the *in vivo* stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems  
15 including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded  
20 by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., Current Op. Biotech., 1995, 6, 698-708).

The pharmaceutical compositions of the present invention may be administered in a number of ways depending  
25 upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous drip,  
30 subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be  
35 particularly useful for oral administration.

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Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or 5 oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-10 aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, 15 diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the 20 invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents, particularly those used for tumor and cancer treatment. Examples of such 25 chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin 30 D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6- 35 mercaptopurine, 6-thioguanine, cytarabine (CA), 5-

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azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUDR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide, 5 trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed., 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used 10 individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and 15 oligonucleotide).

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with 20 the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can 25 easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC<sub>50</sub>s found to be effective *in vitro* and in *in vivo* animal models. In 30 general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence 35 times and concentrations of the drug in bodily fluids or

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tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, 5 ranging from 0.01 µg to 100 g per kg of body weight, once or more daily, to once every 20 years.

Thus, in the context of this invention, by "therapeutically effective amount" is meant the amount of the compound which is required to have a therapeutic effect 10 on the treated individual. This amount, which will be apparent to the skilled artisan, will depend upon the age and weight of the individual, the type of disease to be treated, perhaps even the gender of the individual, and other factors which are routinely taken into consideration 15 when designing a drug treatment. A therapeutic effect is assessed in the individual by measuring the effect of the compound on the disease state in the animal. For example, if the disease to be treated is cancer, therapeutic effects are assessed by measuring the rate of growth or the size of 20 the tumor, or by measuring the production of compounds such as cytokines, production of which is an indication of the progress or regression of the tumor.

The following examples illustrate the present invention and are not intended to limit the same.

25

#### EXAMPLES

##### **EXAMPLE 1: Synthesis of Oligonucleotides**

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by 30 iodine.  $\beta$ -cyanoethyldiisopropyl-phosphoramidites are purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of  $^3\text{H}-1,2\text{-}$  benzodithiole-3-one 1,1-dioxide in acetonitrile for the 35 stepwise thiation of the phosphite linkages. The thiation

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cycle wait step was increased to 68 seconds and was followed by the capping step. Cytosines may be 5-methyl cytosines. (5-methyl deoxycytidine phosphoramidites available from Glen Research, Sterling, VA or Amersham 5 Pharmacia Biotech, Piscataway, NJ)

2'-methoxy oligonucleotides are synthesized using 2'-methoxy  $\beta$ -cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after 10 pulse delivery of tetrazole and base is increased to 360 seconds. Other 2'-alkoxy oligonucleotides are synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2' -fluoro oligonucleotides are synthesized as described in Kawasaki et al. (J. Med. Chem., 1993, 36, 831-841). Briefly, the protected nucleoside N<sup>6</sup>-benzoyl-2'-deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9- $\beta$ -D-arabinofuranosyladenine as 20 starting material and by modifying literature procedures whereby the 2'- $\alpha$ -fluoro atom is introduced by a S<sub>N</sub>2-displacement of a 2'- $\beta$ -O-trifyl group. Thus N<sup>6</sup>-benzoyl-9- $\beta$ -D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) 25 intermediate. Deprotection of the THP and N<sup>6</sup>-benzoyl groups is accomplished using standard methodologies and standard methods are used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine is 30 accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9- $\beta$ -D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to 35 give diisobutyryl di-THP protected arabinofuranosylguanine.

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Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

5       Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a known procedure in which 2, 2'-anhydro-1- $\beta$ -D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

10      2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N<sup>4</sup>-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

15      2'-(2-methoxyethyl)-modified amidites were synthesized according to Martin, P. (Helv. Chim. Acta, 1995, 78, 486-506). For ease of synthesis, the last nucleotide may be a deoxynucleotide. 2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>.cytosines may be 5-methyl cytosines.

20      **Synthesis of 5-Methyl cytosine monomers:**

2,2'-Anhydro[1-( $\beta$ -D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasaki, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to

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give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

5        2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the  
10 solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel  
15 column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

20 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2' -O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the  
25 mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The  
30 solvent was evaporated and triturated with CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5 L) and extracted with 2x500 mL of saturated NaHCO<sub>3</sub> and 2x500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. 275 g of residue was obtained. The residue  
35 was purified on a 3.5 kg silica gel column, packed and

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eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

5 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and 10 acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture 15 evaporated at 35°C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl<sub>3</sub>. The combined organics were dried with sodium sulfate and 20 evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

25 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a 30 solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture 35 stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later

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solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble 5 solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

10 A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). 15 The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue 20 was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

25 N<sup>4</sup>-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine:

2' -O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the 30 reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO<sub>4</sub> and evaporated to give a residue (96 35 g). The residue was chromatographed on a 1.5 kg silica

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column using EtOAc/Hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N<sup>4</sup>-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-

5   methylcytidine-3'-amidite:

N<sup>4</sup>-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added 10 with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-15 extracted with CH<sub>2</sub>Cl<sub>2</sub> (300 mL), and the extracts were combined, dried over MgSO<sub>4</sub> and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title 20 compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al., Nucl. Acids Res., 1993, 21, 3197-3203) using commercially available phosphoramidites (Glen 25 Research, Sterling VA or ChemGenes, Needham MA).

**2'-O-(dimethylaminoxyethyl) nucleoside amidites**

2'-(Dimethylaminoxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminoxyethyl) nucleoside amidites] are prepared as described in the following 30 paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

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5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine

O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for

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ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a  
5 warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was  
10 purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield  
15 based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

20        5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxypthalimide (7.24g, 44.36mmol). It was then dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40°C. The  
25 reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red  
30 coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a  
35 flash column and eluted with ethyl acetate:hexane (60:40),

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to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylidiphenylsilyl-5-methyluridine as white foam (21.819, 86%).

5 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylidiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 hr the mixture was 10 filtered, the filtrate was washed with ice cold CH<sub>2</sub>Cl<sub>2</sub>, and the combined organic phase was washed with water, brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to get 2'-O-(aminoxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% 15 aqueous solution, w/w, 1.1eg.) was added and the mixture for 1 hr. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy) ethyl]-5-methyluridine as white foam (1.95, 78%).

20 5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this 25 solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 hr, the reaction monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in 30 MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was 35

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added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

15 2'-O-(dimethylaminoxyethyl)-5-methyluridine  
Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>, to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

25 5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine  
2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum

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and the residue chromatographed and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-

5 5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine

(1.08g, 1.67mmol) was co-evaporated with toluene (20mL).

To the residue N,N-diisopropylamine tetrazonide (0.29g,

10 1.67mmol) was added and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-

N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12mL, 6.08mmol)

was added. The reaction mixture was stirred at ambient

15 temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5%

aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over

20 anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam

(1.04g, 74.9%).

25 Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. Patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers 30 containing MMI linkages were synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

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Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al. (Acc. Chem. Res., 1995, 28, 366-374). The amide moiety is readily accessible by simple and well-known synthetic methods and 5 is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and Weller).

10 Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al. (Science, 1991, 254, 1497-1500).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated 15 ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at 20 least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by <sup>31</sup>P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by 25 Chiang et al. (J. Biol. Chem., 1991, 266, 18162). Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

**Example 2: Oligonucleotide Sequences and Cell Culture**

Antisense oligonucleotides were designed to E-selectin, Ha-ras, c-raf, JNK1, and JNK2. Additional 30 sequences were designed as scrambled controls. The sequence of the oligonucleotides used are given in Table 1. All of these oligonucleotides except ISIS 11928 (SEQ ID NO. 1) contain 2'-O-methoxyethyl/phosphodiester residues 35 flanking a 2'-deoxynucleotide/phosphorothioate region.

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ISIS 11928 (SEQ ID NO. 1) is a fully phosphorothioated oligonucleotide with all 2'-methoxyethoxy nucleotides, except for a 3' terminal 2'-deoxy nucleotide. The target sequence for E-selectin was obtained from the Genbank endothelial leukocyte adhesion molecule I exon 1 sequence, HUMELAM1 (Accession number M61895; SEQ ID NO. 8). The target sequence for Ha-ras was obtained from the Genbank Ha-ras sequence, HSRAS1 (Accession number V00574; SEQ ID NO. 10). The target sequence for *c*-raf was obtained from the Genbank *c*-raf sequence, HSRAFR (Accession number X03484; SEQ ID NO. 12). The target sequence for JNK1 was obtained from the Genbank JNK1 sequence, HUMJNK1 (Accession number L26318; SEQ ID NO. 14). The target sequence for JNK2 was obtained from the Genbank JNK2 sequence, HUMJNK2 (Accession number L31951; SEQ ID NO. 16).

TABLE 1  
Nucleotide Sequences of Mixed Backbone Chimeric (deoxy gapped) 2'-O-methoxyethyl Oligonucleotides

5' ISIS NO.	NUCLEOTIDE SEQUENCE (5' -> 3') <sup>1</sup>	SEQ NO:	ID	TARGET GENE
SEQ				
11928	<u>G</u> sA <u>A</u> s <u>G</u> sTs <u>C</u> sAs <u>G</u> s <u>C</u> sAs <u>A</u> s <u>G</u> s <u>A</u> s <u>C</u> sAs <u>G</u> s <u>C</u> sT	1		E-selectin
12854	<u>T</u> o <u>C</u> o <u>C</u> o <u>G</u> o <u>C</u> s <u>C</u> ts <u>G</u> sTs <u>G</u> sAs <u>C</u> s <u>A</u> st <u>G</u> o <u>C</u> o <u>A</u> o <u>T</u> o <u>T</u>	2		c-raf
15727	<u>A</u> o <u>T</u> o <u>G</u> o <u>C</u> o <u>A</u> o <u>T</u> ts <u>S</u> cs <u>C</u> ts <u>G</u> s <u>C</u> sc <u>S</u> cs <u>C</u> o <u>A</u> o <u>G</u> o <u>G</u> o <u>A</u>	3		12854 control
10	<u>T</u> o <u>C</u> o <u>C</u> g <u>S</u> ts <u>C</u> as <u>S</u> ts <u>C</u> ts <u>G</u> s <u>C</u> ts <u>S</u> cs <u>C</u> o <u>T</u> o <u>C</u> o <u>A</u> o <u>G</u> o <u>G</u> o <u>G</u>	4		Ha-ras
17552	<u>T</u> o <u>C</u> o <u>A</u> as <u>G</u> sts <u>A</u> sts <u>S</u> as <u>G</u> s <u>C</u> cs <u>C</u> o <u>A</u> o <u>C</u> o <u>A</u> o <u>T</u> o <u>G</u> o <u>G</u>	5		15168 Control
15347	<u>C</u> o <u>T</u> o <u>C</u> o <u>T</u> o <u>C</u> o <u>T</u> ts <u>G</u> sts <u>A</u> sg <u>S</u> gs <u>C</u> sc <u>S</u> cs <u>G</u> s <u>C</u> o <u>T</u> o <u>T</u> o <u>G</u> o <u>G</u>	6		JNK1
15354	<u>G</u> o <u>T</u> o <u>C</u> o <u>C</u> o <u>G</u> o <u>G</u> cs <u>G</u> cs <u>C</u> as <u>G</u> s <u>C</u> sc <u>S</u> cs <u>A</u> o <u>A</u> o <u>G</u> o <u>T</u> o <u>C</u>	7		JNK2

15 <sup>1</sup> Underlined residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines are 5-methyl-cytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

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Human dermal microvascular cells (HMVEC-d; Clonetics, San Diego, CA) were cultivated in endothelial basal media (EBM, Clonetics) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were grown in 100 mm petri dishes until 70-80% confluent, then washed with PBS and OPTI-MEM (Life Technologies, Inc., Gaithersburg, MD). The cells were then incubated in the presence of OPTI-MEM and 3 mg/ml LIPOFECTIN (a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water (Life Technologies, Gaithersburg, MD), per 100 nM of oligonucleotide followed by addition of oligonucleotide at the appropriate concentrations.

For determination of mRNA levels by Northern blot, total RNA was prepared from cells by the guanidinium isothiocyanate procedure or by the Qiagen RNAEASY method (Qiagen, Santa Clarita, CA) at the indicated times after initiation of oligonucleotide treatment. Northern blot analysis was performed as described in Current Protocols in Molecular Biology (Ausubel, F.M., et al., (eds), 1987, Greene Publishing Assoc. and Wiley Interscience, New York). A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe was purchased from Clontech (Palo Alto, CA, Catalog Number 9805-1). RNA was quantified and normalized to G3PDH mRNA levels using a Molecular Dynamic PHOSPHORIMAGER (Sunnyvale, CA).

For determination of protein levels by Western blot, cellular extracts were prepared using 300 ml of RIPA extraction buffer per 100-mm dish. The protein concentration was quantified by Bradford assay using the BioRad kit (BioRad, Hercules, CA). Equal amounts of protein were loaded on 10% or 12% SDS-PAGE mini-gel (Novex, San Diego, CA). Once transferred to PVDF membranes (Millipore, Bedford, MA), the membranes were then treated

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for a minimum of 2 hours with specific primary antibody followed by incubation with secondary antibody conjugated to HRP. The results were visualized by Enhanced Chemiluminescent (ECL) detection (New England BioLab, Beverly, MA). In some experiments, the blots were stripped in stripping buffer (2% SDS, 12.5 mM Tris, pH 6.8) for 30 minutes at 50°C. After extensive washing, the blots were blocked and blotted with different primary antibody.

5           **Example 3: Inhibition of c-raf and Ha-ras Expression by  
10 Antisense Oligonucleotides**

ISIS 12854 (SEQ ID NO. 2) is a 2'-O-methoxyethyl mixed backbone chimeric antisense oligonucleotide designed to hybridize with 3'-untranslated sequences contained within human c-raf mRNA. To determine whether this antisense  
15 oligonucleotide is effective as a c-raf inhibitor in endothelial cells, human dermal microvascular cells (HMVEC) were treated with ISIS 12854 (SEQ ID NO. 2) as described in Example 2. C-raf mRNA and protein expression was examined by northern and Western blot analysis. The c-raf cDNA probe was generated from the plasmid, p627 (American Type Culture Collection, Manassas, VA; catalog #41050), following the supplied instructions. The c-raf antibody was obtained from Transduction Laboratories, Inc. (Lexington, KY). Northern blot results are shown in Table  
20 2. Western blot results are shown in Figure 1.

25           **TABLE 2**  
**Dose Response of ISIS 12854 on c-raf mRNA Levels in HMVEC Cells**

30	<b>ISIS #</b>	<b>SEQ ID NO:</b>	<b>ASO Gene Target</b>	<b>Dose</b>	<b>% mRNA Expression</b>	<b>% mRNA Inhibition</b>
	LIPOFECTIN	---	---	---	100%	---
	15727	3	control	100 nM	96%	4%

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	"	"	"	200 nM	135%	---
	12854	2	<i>c-raf</i>	0.5 nM	67%	33%
	"	"	"	2.5 nM	31%	69%
	"	"	"	10 nM	15%	85%
5	"	"	"	50 nM	10%	90%
	"	"	"	100 nM	13%	87%
	"	"	"	200 nM	3%	97%

Treatment of HMVEC with ISIS 12854 (SEQ ID NO. 2)

10 resulted in dramatically reduced *c-raf* mRNA levels. Reduction of *c-raf* mRNA levels was dose-dependent in the range of 0.5 to 200 nM. The IC<sub>50</sub> for *c-raf* mRNA reduction was approximately 2.5 nM. The control oligonucleotide, ISIS 15727 (SEQ ID NO. 3) did not exhibit any effect on *c-raf* mRNA.

15 Reduction of *c-raf* protein levels also occurred following oligonucleotide treatment. Reduction in protein expression was first detectable 24 hours after treatment and maximal reduction of *c-raf* protein levels was achieved 20 48 hours after treatment with 150 nM oligonucleotide.

Reduction of *c-raf* protein levels persisted up to 72 hour following initial treatment. Inhibition of *c-raf* expression by ISIS 12854 (SEQ ID NO. 2) was specific since A-raf protein expression was largely unaffected when the 25 same blot was stripped and blotted with antibody against A-raf (Transduction Laboratories, Inc., Lexington, KY).

ISIS 15168 (SEQ ID NO. 4) is a 2'-O-methoxyethyl mixed backbone chimeric antisense oligonucleotide designed to hybridize with sequences contained within the human Ha-ras 30 mRNA. To determine the effect of ISIS 15168 (SEQ ID NO. 4) on Ha-ras expression in endothelial cells, HMVEC were treated, as described in Example 2, with oligonucleotide and northern blotting was used to measure Ha-ras mRNA levels. A Ha-ras probe was generated from the plasmid,

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pbc-N1 (American Type Culture Collection, Manassas, VA; catalog #41001), following the supplied instructions. Results are shown in Table 3.

**TABLE 3**

**5 Dose Response of Ha-ras Antisense Oligodeoxynucleotides on  
Ha-ras mRNA Levels in HMVEC Cells**

		SEQ ID	ASO Gene		% mRNA	% mRNA
	ISIS #	NO:	Target	Dose	Expression	Inhibition
	LIPOFECTIN™	---	---	---	100%	---
10	15168	4	Ha-ras	1 nM	61%	39%
		"	"	5 nM	44%	56%
		"	"	25 nM	19%	81%
		"	"	50 nM	10%	90%
		"	"	100 nM	9%	91%
15	17552	5	control	1 nM	103%	---
		"	"	5 nM	115%	---
		"	"	25 nM	96%	4%
		"	"	50 nM	109%	---
		"	"	100 nM	105%	---

20

Reduction of Ha-ras mRNA levels in HMVEC was observed following oligonucleotide treatment and was found to be sequence-specific, and dose-dependent ( $IC_{50} < 5$  nM).

25 To examine the effect of ISIS 15168 (SEQ ID NO. 4) on Ha-ras protein levels, total ras protein was immunoprecipitated using a pan-ras monoclonal antibody (Oncogene Science, Cambridge, MA). The precipitated proteins were analyzed by SDS-PAGE, and Ha-ras protein levels were determined by western blot using a monoclonal antibody specific for Ha-ras (Oncogene Science, Cambridge, MA). As shown in Figure 2, dose-dependent reduction of Ha-ras protein expression was observed 48 hours following treatment with ISIS 15168 (SEQ ID NO. 4). This reduction

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persisted up to 72 hours following initial treatment. The kinetics of Ha-ras reduction was slower than that of c-raf. The control oligonucleotide , ISIS 17552 (SEQ ID NO. 5), had no effect on Ha-ras protein level. The same blot was  
5 stripped and blotted a second time with a Ki-ras-specific antibody (Oncogene Science, Cambridge, MA). No effect on Ki-ras protein levels was observed in cells treated with either ISIS 15168 (SEQ ID NO. 4) or the control oligonucleotide, ISIS 17552 (SEQ ID NO. 5).

10 **Example 4: Effect of inhibiting c-raf and Ha-ras Gene Expression on the Induction of E-selectin**

The effect of c-raf and Ha-ras antisense oligonucleotide treatment on the induction of E-selectin by TNF- $\alpha$  was examined. HMVEC cells were treated with either  
15 the c-raf or Ha-ras antisense compound under dose-response conditions or over time at a single dose level followed by stimulation of E-selectin expression by TNF- $\alpha$  for 5 hours. The cell surface expression of E-selectin was determined by flow cytometry analysis. Following oligonucleotide  
20 treatment, cells were detached from the plates and analyzed for surface expression of cell adhesion molecules using a Becton Dickinson (San Jose, CA) FACScan. TNF- $\alpha$  and FITC conjugated antibody for E-selectin were obtained from R & D Systems (Minneapolis, MN). PE-conjugated antibody for  
25 ICAM-1 was obtained from Pharmingen (San Diego, CA). VCAM-1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Cell surface expression was calculated using the mean value of fluorescence intensity using 3,000-5,000 cells stained with the appropriate antibody for each  
30 sample and time point. Results are expressed as percentage of control (cell surface expression induced by TNF- $\alpha$  in cells that were not treated with oligonucleotides) based upon mean fluorescence intensity. Results are shown in Tables 4 and 5. Basal expression of E-selectin and VCAM-1

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is undetectable in the absence of TNF- $\alpha$  whereas a low level of basal expression is detectable for ICAM-1.

**ABLE 4**

**Dose Response of the effect of c-raf and Ha-ras Antisense**

5                   **Oligonucleotides in Induction of E-selectin**

	ISIS #	SEQ ID	ASO Gene	Dose	% Cell	% Cell
		NO:	Target		Surface Expression	Surface Inhibition
	LIPOFECTIN™	---	---	---	100%	---
10	12854	2	c-raf	25 nM	37%	63%
	"	"	"	75 nM	26%	74%
	"	"	"	150 nM	23%	77%
15	15727	3	control	25 nM	119%	---
	"	"	"	75 nM	94%	6%
	"	"	"	150 nM	106%	---
	15168	4	Ha-ras	25 nM	72%	28%
	"	"	"	75 nM	47%	53%
20	"	"	"	150 nM	41%	59%
	17552	5	control	25 nM	134%	---
	"	"	"	75 nM	116%	---
	"	"	"	150 nM	116%	---

Dose-dependent inhibition of E-selectin cell surface expression was observed in cells treated with both antisense compounds, ISIS 12854 (SEQ ID NO. 2), targeted to c-raf and ISIS 15168 (SEQ ID NO. 4) targeted to Ha-ras.

Maximal inhibition of E-selectin induction was approximately 80% for ISIS 12854 (SEQ ID NO. 2) and 60% for ISIS 15168 (SEQ ID NO. 4). Control oligonucleotides (SEQ ID NO. 3 and SEQ ID NO. 5) exhibited little to no effect on E-selectin induction. These results indicate that reduction of c-raf or Ha-ras protein levels blocked the induction of E-selectin by TNF- $\alpha$  in HMVEC.

The effects of c-raf, Ha-ras, and a E-selectin antisense oligonucleotide on TNF- $\alpha$ -induced E-selectin cell

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surface expression under kinetic conditions were examined. If the inhibition of E-selectin induction by the *c-raf* and Ha-ras antisense compounds was dependent on the reduction of *c-raf* and Ha-ras protein levels, the kinetics of inhibition of E-selectin induction by the antisense compounds should correlate with suppression of *c-raf* and Ha-ras protein levels (which is dependent on the half-lives of the proteins in cells). Inhibition of E-selectin induction using the E-selectin antisense oligonucleotide should occur much more quickly since this inhibitor blocks E-selectin induction directly. To test this, cells were treated, separately, with antisense oligonucleotides to E-selectin, ICAM-1 and VCAM-1 and allowed to recover prior to TNF- $\alpha$  treatment. TNF- $\alpha$  was added at different time points between 12 and 72 hours following oligonucleotide treatment. E-selectin cell surface expression was measured by flow cytometry analysis after 5 hours of TNF- $\alpha$  treatment. Results are shown in Table 5.

TABLE 5

20 Time Course of the Effect of *c-raf* and Ha-ras Antisense Oligonucleotides in Induction of E-selectin

		SEQ ISIS #	ID NO:	ASO Gene Target	Time (hours)	% Cell Surface Expression	% Cell Surface Inhibition
		LIPOFECTIN	---	---	---	100%	---
25	11928	1	E-	E-selectin	12h/20h	22%	78%
	"	"	"	"	48h	29%	71%
	"	"	"	"	72h	32%	68%
	12854	2	<i>c-raf</i>	<i>c-raf</i>	12h/20h	58%	42%
	"	"	"	"	48h	23%	77%
30	"	"	"	"	72h	24%	76%
	15727	3	control	control	12h/20h	93%	7%

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	"	"	"	48h	106%	---
	"	"	"	72h	81%	19%
	15168	4	Ha-ras	12h/20h	87%	13%
	"	"	"	48h	42%	58%
5	"	"	"	72h	42%	58%
	17552	5	control	12h/20h	113%	---
	"	"	"	48h	116%	---
	"	"	"	72h	111%	---

10           Concentrations as low as 20 nM of the E-selectin oligonucleotide, ISIS 11928 (SEQ ID NO. 1) were found to block 80% of E-selectin cell surface expression 12 hours following treatment. In contrast, maximal inhibition of E-selectin induction by *c-raf* and Ha-ras antisense compounds, 15 SEQ ID NO. 2 and SEQ ID NO. 4, respectively, was observed 48 hours following antisense treatment. Some inhibition of E-selectin induction was observed at 12 hours following treatment, but this inhibition was clearly not maximal. These results strongly suggest that inhibition of E-selectin induction by TNF- $\alpha$  in HMVEC following treatment with *c-raf* and Ha-ras antisense oligonucleotides is a consequence of reduced *c-raf* and Ha-ras protein levels.

20           **Example 5: Effect of *c-raf* and Ha-ras Antisense Oligonucleotides on the Induction of other Cell Adhesion Molecules**

25           TNF- $\alpha$  induction of ICAM-1 and VCAM-1 in cells treated with *c-raf* and Ha-ras antisense oligonucleotides was also examined to further define the roles of *c-raf* and Ha-ras in cytokine signaling. Oligonucleotides were tested, using flow cytometry analysis, as described in Example 4. Results are shown in Table 6 (ICAM-1) and Table 7 (VCAM-1).

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TABLE 6

**Dose response of the effect of *c-raf* and *Ha-ras* antisense oligonucleotides in induction of ICAM-1**

5	ISIS #	SEQ	ASO	% Cell		% Cell
		ID	Gene	Dose	Surface	Surface
		NO:	Target	(conc.)	Expression	Inhibition
	LIPOFECTIN™	---	---	---	100%	---
	12854	2	<i>c-raf</i>	20 nM	80%	20%
	"	"	"	50 nM	62%	38%
	"	"	"	100 nM	57%	43%
10	15727	3	control	20 nM	97%	3%
	"	"	"	50 nM	89%	11%
	"	"	"	100 nM	87%	13%
	15168	4	<i>Ha-ras</i>	20 nM	81%	19%
	"	"	"	50 nM	62%	38%
15	"	"	"	100 nM	54%	46%
	17552	5	control	20 nM	102%	---
	"	"	"	50 nM	100%	---
	"	"	"	100 nM	98%	2%

20        Induction of ICAM-1 by TNF- $\alpha$  was also blocked by *c-raf* (ISIS 12854, SEQ ID NO. 2) and *Ha-ras* (ISIS 15168, SEQ ID NO. 4) antisense oligonucleotides, with maximal inhibition greater than 40%.

25        Treatment of cells with *c-raf* and *Ha-ras* antisense oligonucleotides inhibited VCAM-1 expression in a dose-dependent fashion as well. Results are shown in Table 7.

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TABLE 7

**Dose response of the effect of *c-raf* and *Ha-ras* antisense oligonucleotides in induction of VCAM-1**

5	ISIS #	SEQ	ASO	% Cell		% Cell
		ID	Gene	Dose	Surface	Surface
		NO:	Target	(conc.)	Expression	Inhibition
	LIPOFECTIN™	---	---	---	100%	---
	12854	2	<i>c-raf</i>	20 nM	46%	54%
	"	"	"	50 nM	37%	63%
	"	"	"	100 nM	28%	72%
10	15727	3	control	20 nM	69%	31%
	"	"	"	50 nM	81%	19%
	"	"	"	100 nM	74%	26%
	15168	4	<i>Ha-ras</i>	20 nM	70%	30%
	"	"	"	50 nM	51%	49%
15	"	"	"	100 nM	44%	56%
	17552	5	control	20 nM	111%	---
	"	"	"	50 nM	97%	3%
	"	"	"	100 nM	85%	15%

20           Induction of VCAM-1 by TNF- $\alpha$  was also blocked by *c-raf* (ISIS 12854, SEQ ID NO. 2) and *Ha-ras* (ISIS 15168, SEQ ID NO. 4) antisense oligonucleotides. Maximum inhibition for ISIS 12854 (SEQ ID NO. 2) was greater than 70%, while maximum inhibition for ISIS 15168 (SEQ ID NO. 4) was greater than 50%.

25           **Example 6: Effect of *c-raf* and *Ha-ras* Antisense Oligonucleotides on Cell Adhesion Molecule mRNA levels**

30           Northern blot analysis was carried out to examine whether *c-raf* and *Ha-ras* antisense oligonucleotides inhibit the induction of cell adhesion molecules at the transcriptional level. Cells were treated with *c-raf* (ISIS

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12854, SEQ ID NO. 2) or Ha-ras (ISIS 15168, SEQ ID NO. 4) antisense oligonucleotides and allowed to recover for 48 hrs. TNF- $\alpha$  was added two to three hours prior to RNA analysis and Northern blotting was performed with probes 5 specific for E-selectin, ICAM-1, and VCAM-1. The probe for E-selectin was obtained by PCR amplification using primers directed to HUMELAM1A (Genbank Accession No. M24736; SEQ ID NO. 18).

The probe for ELAM-1 was obtained by PCR amplification 10 using the following primers:

FORWARD 5'-TTGAAGTCATGATTGCTTCACAGTT-3' SEQ ID NO. 20

REVERSE 5'-TTCTGATTCTTTGAACTTAAAGGAT-3' SEQ ID NO. 21

The probe for ICAM-1 was obtained by PCR amplification using the following primers:

15 FORWARD 5'-CGCGGATCCCGTACTCAGAGTT-3' SEQ ID NO. 22

REVERSE 5'-CGGAATTCCGTTCAGGGAGGCGT-3' SEQ ID NO. 23

The probe for VCAM-1 was obtained by PCR amplification using the following primers:

FORWARD 5'-CTTAAAATGCCTGGGAAGATGGTCGT-3' SEQ ID NO. 24

20 REVERSE 5'-ATCAAAGCATTAGCTACACTTTGATT-3' SEQ ID NO. 25

Results are shown in Table 8.

**TABLE 8**

**Effect of c-raf and Ha-ras antisense oligonucleotides on induction of E-selectin, VCAM-1, and ICAM-1**

25	ISIS #	SEQ	ASO	Cell	% mRNA	% mRNA
		ID	Gene	adhesion	Expression	Inhibition
	NO:	Target	molecule			
	LIPOFECTIN™	---	---	---	100%	---
	12854	2	c-raf	E-selectin	22%	78%
	"	"	"	ICAM-1	78%	22%
30	"	"	"	VCAM-1	35%	65%
	15727	3	control	E-selectin	103%	---

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	"	"	"	ICAM-1	107%	---
	"	"	"	VCAM-1	113%	---
	15168	4	Ha-ras	E-selectin	13%	87%
	"	"	"	ICAM-1	73%	27%
5	"	"	"	VCAM-1	29%	71%
	17552	5	contro	E-selectin	92%	8%
			1			
	"	"	"	ICAM-1	101%	---
	"	"	"	VCAM-1	111%	---

10        Induction of mRNA expression of the three cell adhesion molecules was attenuated by both antisense oligonucleotides, whereas treatment with control oligonucleotides exhibited little to no effect. The level of inhibition for each cell adhesion molecule at the mRNA 15 level was consistent with the effects of *c-raf* and Ha-ras antisense treatment on the cell surface expression of cell adhesion molecules.

**Example 7: Effect of *c-raf* Antisense Oligonucleotides on MAP Kinase Activities**

20        To examine the effect of the *c-raf* antisense oligonucleotide (ISIS 12854, SEQ ID NO. 2) on ERK, JNK, and p38 MAPK activities stimulated by TNF- $\alpha$ , *in vitro* kinase assays were performed on extracts derived from cells treated with ISIS 12854 (SEQ ID NO. 2). Cells were treated 25 with oligonucleotides and induced with cytokines. At the indicated time, cells were lysed on ice and debris was removed by centrifugation. Protein concentration was measured by Bradford assay. Lysate containing equal amounts of protein were incubated with primary antibody- 30 agarose conjugates (ERK and p38 assay; Santa Cruz Biotechnology, Santa Cruz, CA), or with JNK1-specific or JNK2-specific antibodies (JNK assay; Upstate Biotechnology, Lake Placid, NY), overnight at 4°C.

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For isoform-specific JNK assays, anti-rabbit IgG conjugated with agarose beads was added to cell extracts following JNK antibody treatment and wash steps and incubated for 2 hours at 4°C. After washing with lysis buffer and kinase buffer, the pelleted beads were incubated with 1 $\mu$ g of substrate (Elk-1 for ERK, ATF-2 for p38, and c-Jun for JNK MAPK) and 100  $\alpha$ M of ATP for 20 minutes at 37°C. MAPK and JNK assay kits, ATF-2 fusion protein, and antibodies for ATF-2, Elk-1 phospho-ATF-2 were purchased from New England Biolabs (Boston, MA). Reactions were terminated by the addition of 3x SDS sample buffer followed by boiling. The samples were loaded on 12% SDS-PAGE gels. Western blot with antibodies specific for phosphorylated substrates (New England Biolabs) was carried out. The results were visualized by ECL.

Cells were treated with the *c-raf* antisense compound, ISIS 12854 (SEQ ID NO. 2), allowed to recover for 48 hours, at which time TNF- $\alpha$  was added for 5 or 15 minutes prior to cell lysis and initiation of the kinase assays. Specific antibody-conjugated agarose beads were used to immunoprecipitate ERK and p38 MAPK, and c-Jun-conjugated agarose beads were used to precipitate JNK. Suitable substrates and ATP were added to the immunoprecipitated kinase complexes and the reaction mixes were analyzed on SDS-PAGE. Western blotting with antibodies specific for phosphorylated substrates was carried out to determine relative kinase activity. Results are shown in Figure 3.

All three kinases were activated by TNF- $\alpha$  after a 15 minutes incubation, as indicated by the heavy phosphorylation of the three substrates. Inhibition of *c-raf* levels by ISIS 12854 (SEQ ID NO. 2) resulted in reduced ERK activity. Surprisingly, JNK activity was also inhibited by treating cells with ISIS 12854 (SEQ ID NO. 2). Activation of p38 MAPK was not affected by *c-raf* antisense treatment.

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These results demonstrate for the first time that *c-raf* inhibition blocks TNF- $\alpha$ -mediated induction of cell adhesion molecules by suppressing the JNK pathway.

5 Antisense oligonucleotides targeted against JNK1 (SEQ ID NO. 6) or JNK2 (SEQ ID NO. 7) were tested for their abilities to inhibit JNK expression, JNK activity and E-selectin induction by TNF- $\alpha$ .

Oligonucleotide treatment, RNA isolation and Northern blots were performed as described in Example 2. A cDNA clone of 10 JNK1 (Derijard et al., *Cell*, 1994, 76, 1025) was radiolabeled and used as a JNK1-specific probe. A cDNA clone of JNK2 (Kallunki et al., *Genes & Development*, 1994, 8, 2996) was radiolabeled and used as a JNK2-specific probe. JNK1 and JNK2 antisense treatment resulted in 15 nearly complete inhibition of JNK1 and JNK2 mRNA expression, respectively, as shown in Figure 4.

Furthermore, both antisense oligonucleotides were isoform-specific at the employed concentrations. The JNK2 antisense molecule will inhibit JNK1 expression slightly at 20 higher oligonucleotide concentrations due to the fact that it is complementary to JNK1 mRNA in 17 of its 20 bases. However, at the concentration tested, the JNK2 antisense oligonucleotide specifically inhibits JNK2 expression without affecting JNK1 levels. Treatment of cells with 25 either JNK1 or JNK2 antisense effectively reduced TNF- $\alpha$ -mediated induction of JNK activity in an isoform-specific manner, as shown in Figure 5. However, JNK2 antisense treatment resulted in substantially greater inhibition of TNF- $\alpha$ -mediated induction of E-selectin cell surface 30 expression relative to JNK1 antisense treatment. Results are shown in Table 9. These results further confirm that the involvement of *c-raf* in TNF- $\alpha$ -mediated induction of cell adhesion molecules in HMVEC involves the regulation of JNK and that this regulation is believed to be specific for 35 the JNK2 isoform.

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**TABLE 9**

**Dose Response of the Effect of JNK Antisense  
Oligonucleotides in induction of E-selectin**

	<b>ISIS #</b>	<b>SEQ</b>	<b>ASO</b>		<b>% Cell</b>		<b>% Cell</b>
		<b>ID</b>	<b>Gene</b>	<b>Time</b>	<b>Surface</b>	<b>Surface</b>	<b>Inhibition</b>
	LIPOFECTIN	---	---	---	100%	---	---
5	15347	6	JNK1	20 nM	89%	11%	
	"	"	"	50 nM	84%	16%	
	"	"	"	100 nM	82%	18%	
10	15354	7	JNK2	20 nM	32%	68%	
	"	"	"	50 nM	29%	71%	
	"	"	"	100 nM	33%	67%	
	15727	3	control	20 nM	137%	---	
	"	"	"	50 nM	128%	---	
15	"	"	"	100 nM	124%	---	

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**WHAT IS CLAIMED IS:**

1. A method of modulating cell adhesion molecule expression comprising treating a cell expressing a cell adhesion molecule with a specific inhibitor of a Tumor Necrosis Factor alpha signaling molecule selected from the group consisting of Ha-ras, c-raf and JNK2, such that cell adhesion molecule expression is modulated.
- 5  
2. The method of claim 1 wherein said cell adhesion molecule is E-selectin, VCAM-1 or ICAM-1.
- 10  
3. The method of claim 1 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding Tumor Necrosis Factor alpha signaling molecule.
- 15  
4. The method of claim 3 wherein said Tumor Necrosis Factor alpha signaling molecule is Ha-ras or c-raf.
- 20  
5. The method of claim 4 wherein said antisense oligonucleotide is hybridizable to Ha-ras or c-raf.
6. The method of claim 5 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 2 or  
25 SEQ ID NO. 4.
7. The method of claim 6 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.
- 30  
8. The method of claim 6 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

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9. The method of claim 1 wherein said cell adhesion molecule is E-selectin and said Tumor Necrosis Factor alpha signaling molecule is JNK-2.

5 10. The method of claim 9 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding JNK-2.

10 11. The method of claim 10 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 7.

12. The method of claim 11 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

15 13. The method of claim 11 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

20 14. A method of inhibiting expression of a MAP kinase within a cell comprising treating said cell with a specific inhibitor of *c-raf*.

25 15. The method of claim 14 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding *c-raf*.

16. The method of claim 15 wherein said oligonucleotide comprises SEQ ID NO. 2.

30 17. The method of claim 16 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

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18. The method of claim 16 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

5       19. A method of treating a disease or condition associated with altered expression of a cell adhesion molecule comprising administering a specific inhibitor of a Tumor Necrosis Factor alpha signaling molecule selected from the group consisting of Ha-ras, c-raf and JNK2, under  
10      conditions wherein expression of said cell adhesion molecule is modulated.

15      20. The method of claim 19 wherein said disease or condition is an inflammatory or immune disease or condition.

20      21. The method of claim 19 wherein said disease is sepsis, rheumatoid arthritis, inflammatory bowel disease, allergic contact dermatitis, psoriasis, diabetes, Grave's disease, allograft rejection or cancer.

25      22. The method of claim 19 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding Tumor Necrosis Factor alpha signaling molecule.

30      23. The method of claim 22 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 2, SEQ ID NO. 4 or SEQ ID NO. 7.

24. The method of claim 23 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

-65-

25. The method of claim 23 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

5 26. An inhibitor of a Tumor Necrosis Factor alpha signaling molecule selected from the group consisting of Ha-ras, c-raf and JNK2, wherein said inhibitor modulates expression of a cell adhesion molecule.

10 27. The inhibitor of claim 26 wherein said cell adhesion molecule is E-selectin, VCAM-1 or ICAM-1.

15 28. The inhibitor of claim 26 comprising an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding a Tumor Necrosis Factor alpha signaling molecule.

29. The inhibitor of claim 27 wherein said tumor Necrosis Facotr alpha signaling molecule is Ha-ras or c-raf.

20 30. The inhibitor of claim 28 wherein said antisense oligonucleotide is hybridizable to Ha-ras or c-raf.

25 31. The inhibitor of claim 30 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 2 or SEQ ID NO. 4.

30 32. The inhibitor of claim 31 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

33. The inhibitor of claim 31 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

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34. The inhibitor of claim 26 wherein said cell adhesion molecule is E-selectin and said Tumor Necrosis Factor alpha signaling molecule is JNK-2.

5 35. The inhibitor of claim 34 comprising an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding JNK-2.

10 36. The inhibitor of claim 35 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 7.

37. The inhibitor of claim 36 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

15 38. The method of claim 36 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

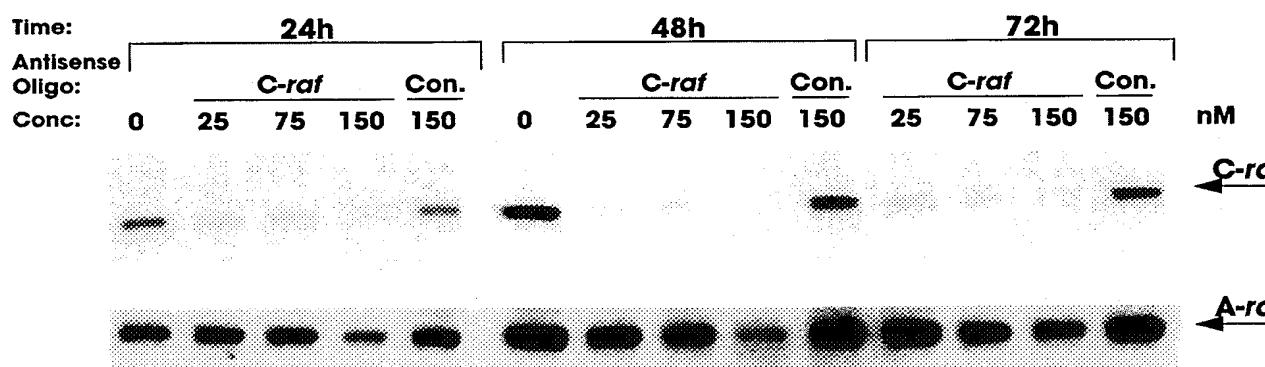


FIG. 1

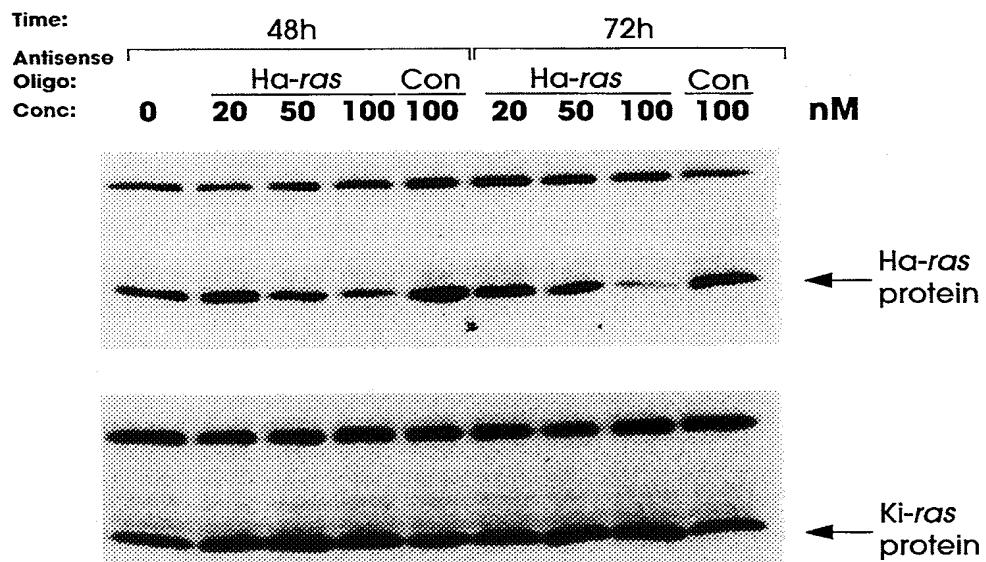


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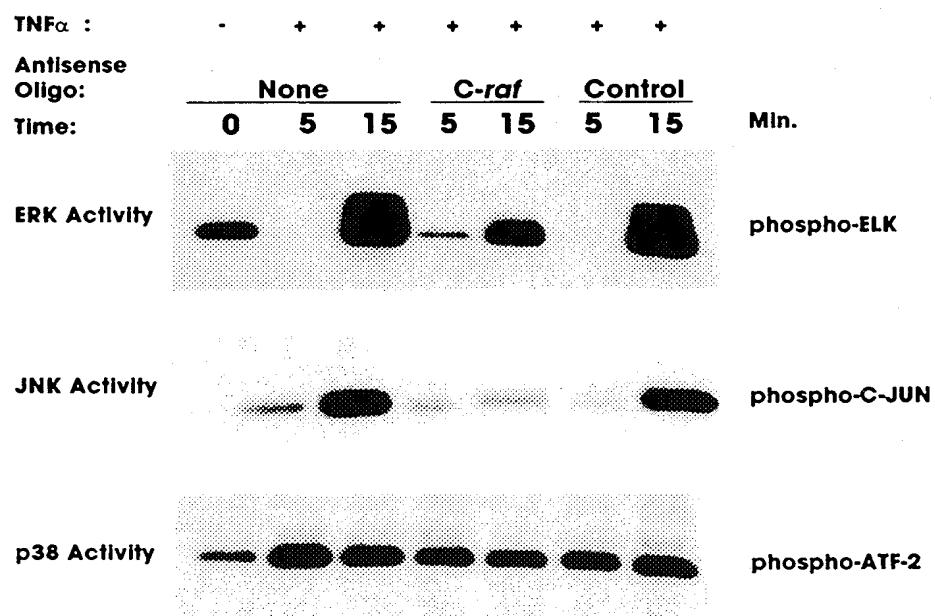


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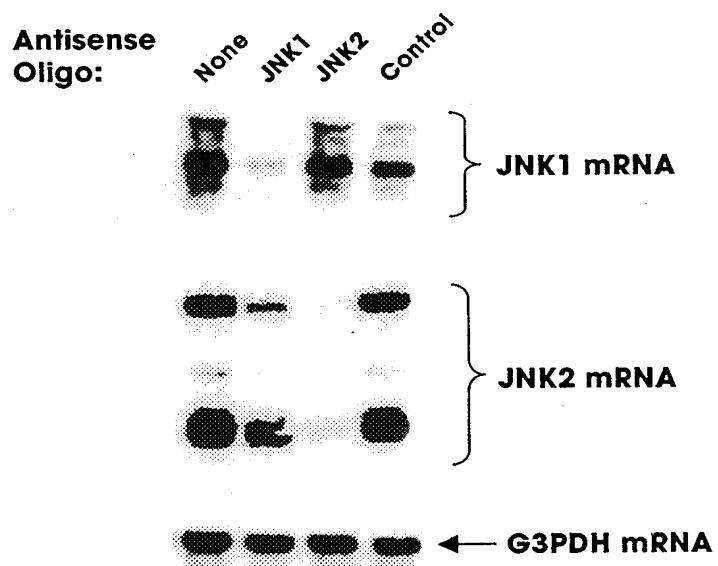


FIG. 4

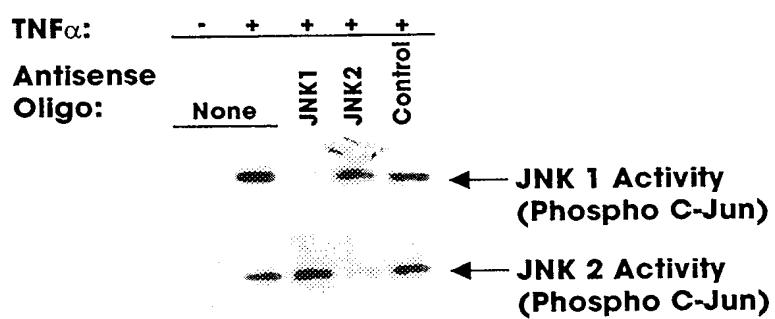


FIG. 5

## SEQUENCE LISTING

<110> Monia, Brett P.  
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Isis Pharmaceuticals, Inc.

<120> METHODS OF MODULATING TUMOR NECROSIS FACTOR  
alpha-INDUCED EXPRESSION OF CELL ADHESION MOLECULES

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ccctggtctg atgcccggagga taccctgca aactgccaat cccagaggac aagactggga 6370

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 Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly  
 35 40 45  
 Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr  
 50 55 60  
 Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys  
 65 70 75 80  
 Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His Gln Tyr  
 85 90 95  
 Arg Glu Gln Ile Lys Arg Val Lys Asp Ser Asp Asp Val Pro Met Val  
 100 105 110  
 Leu Val Gly Asn Lys Cys Asp Leu Ala Ala Arg Thr Val Glu Ser Arg  
 115 120 125  
 Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Tyr Ile Glu Thr  
 130 135 140  
 Ser Ala Lys Thr Arg Gln Gly Val Glu Asp Ala Phe Tyr Thr Leu Val  
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 gctgcata atg gag cac ata cag gga gct tgg aag acg atc acg aat ggt 171  
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 ttt gga ttc aaa gat gcc gtg ttt gat ggc tcc agc tgc atc tct cct 219  
 Phe Gly Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro  
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 aca ata gtt cag cag ttt ggc tat cag cgc cgg gca tca gat gat ggc 267  
 Thr Ile Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly  
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 Lys Leu Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu  
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 Pro Asn Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu  
   65                70                75  
  
 cat gac tgc ctt atg aaa gca ctc aag gtg agg ggc ctg caa cca gag 411  
 His Asp Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu  
   80                85                90  
  
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 Cys Cys Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala  
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 Arg Leu Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu  
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 Arg Lys Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe  
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 His Cys Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile  
   175                180                185                190  
  
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Gln Arg Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu			
255	260	265	270
cct gtg gac agc agg atg att gag gat gca att cga agt cac acg gaa			987
Pro Val Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu			
275	280	285	
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Ser Ala Ser Pro Ser Ala Leu Ser Ser Pro Asn Asn Leu Ser Pro			
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Thr Gly Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg			
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gca cca gta tct ggg acc cag gag aaa aac aaa att agg cct cgt gga			1131
Ala Pro Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly			
320	325	330	
cag aga gat tca agc tat tat tgg gaa ata gaa gcc agt gaa gtg atg			1179
Gln Arg Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met			
335	340	345	350
ctg tcc act cgg att ggg tca ggc tct ttt gga act gtt tat aag ggt			1227
Leu Ser Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly			
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Lys Trp His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro			
370	375	380	
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Thr Pro Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg			
385	390	395	
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Lys Thr Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys			
400	405	410	
gac aac ctg gca att gtg acc cag tgg tgc gag ggc agc agc ctc tac			1419
Asp Asn Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr			
415	420	425	430
aaa cac ctg cat gtc cag gag acc aag ttt cag atg ttc cag cta att			1467
Lys His Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile			
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gac att gcc cgg cag acg gct cag gga atg gac tat ttg cat gca aag			1515
Asp Ile Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys			
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aac atc atc cat aga gac atg aaa tcc aac aat ata ttt ctc cat gaa			1563
Asn Ile Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu			
465	470	475	
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Gly Leu Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser			
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Trp Met Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser	
515 520 525	
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Phe Gln Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met	
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Thr Gly Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile	
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Lys Asn Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys	
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Lys Val Lys Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile	
595 600 605	
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Glu Leu Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu	
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cca tcc ttg cat cgg gca gcc cac act gag gat atc aat gct tgc acg	2043
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625 630 635	
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Leu Thr Thr Ser Pro Arg Leu Pro Val Phe	
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 Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu  
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 Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn  
 50 55 60  
 Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp  
 65 70 75 80  
 Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys  
 85 90 95  
 Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu  
 100 105 110  
 Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val  
 115 120 125  
 Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys  
 130 135 140  
 Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu  
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 Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln  
 180 185 190  
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 Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg  
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 Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val  
 260 265 270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala  
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 325 330 335  
 Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser  
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 Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp  
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 His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro  
 370 375 380  
 Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr  
 385 390 395 400  
 Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn  
 405 410 415  
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 Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu  
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 Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met  
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 Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln  
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 Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Thr Gly  
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 Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn  
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 Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val  
 580 585 590  
 Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu  
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Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser  
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Ser	Val Glu Ile Gly Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln	
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aat	tta aaa cct ata ggc tca gga gct caa gga ata gta tgc gca gct	147
Asn	Leu Lys Pro Ile Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala	
30	35	40

tat	gat gcc att ctt gaa aga aat gtt gca atc aag aag cta agc cga	195
Tyr	Asp Ala Ile Leu Glu Arg Asn Val Ala Ile Lys Lys Leu Ser Arg	
45	50	55

cca	ttt cag aat cag act cat gcc aag cgg gcc tac aga gag cta gtt	243
Pro	Phe Gln Asn Gln Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val	
60	65	70

ctt	atg aaa tgt gtt aat cac aaa aat ata att ggc ctt ttg aat gtt	291
Leu	Met Lys Cys Val Asn His Lys Asn Ile Ile Gly Leu Leu Asn Val	
80	85	90

ttc	aca cca cag aaa tcc cta gaa gaa ttt caa gat gtt tac ata gtc	339
Phe	Thr Pro Gln Lys Ser Leu Glu Glu Phe Gln Asp Val Tyr Ile Val	
95	100	105

atg	gag ctc atg gat gca aat ctt tgc caa gtg att cag atg gag cta	387
Met	Glu Leu Met Asp Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu	
110	115	120

gat	cat gaa aga atg tcc tac ctt ctc tat cag atg ctg tgt gga atc	435
Asp	His Glu Arg Met Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile	
125	130	135

aag	cac ctt cat tct gct gga att att cat cgg gac tta aag ccc agt	483
Lys	His Leu His Ser Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser	
140	145	150

aat ata gta gta aaa tct gat tgc act ttg aag att ctt gac ttc ggt	531
Asn Ile Val Val Lys Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly	
160 165 170	
ctg gcc agg act gca gga acg agt ttt atg atg acg cct tat gta gtg	579
Leu Ala Arg Thr Ala Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val	
175 180 185	
act cgc tac tac aga gca ccc gag gtc atc ctt ggc atg ggc tac aag	627
Thr Arg Tyr Tyr Arg Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys	
190 195 200	
gaa aac gtg gat tta tgg tct gtg ggg tgc att atg gga gaa atg gtt	675
Glu Asn Val Asp Leu Trp Ser Val Gly Cys Ile Met Gly Glu Met Val	
205 210 215	
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Cys His Lys Ile Leu Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn	
220 225 230 235	
aaa gtt att gaa cag ctt gga aca cca tgt cct gaa ttc atg aag aaa	771
Lys Val Ile Glu Gln Leu Gly Thr Pro Cys Pro Glu Phe Met Lys Lys	
240 245 250	
ctg caa cca aca gta agg act tac gtt gaa aac aga cct aaa tat gct	819
Leu Gln Pro Thr Val Arg Thr Tyr Val Glu Asn Arg Pro Lys Tyr Ala	
255 260 265	
gga tat agc ttt gag aaa ctc ttc cct gat gtc ctt ttc cca gct gac	867
Gly Tyr Ser Phe Glu Lys Leu Phe Pro Asp Val Leu Phe Pro Ala Asp	
270 275 280	
tca gaa cac aac aaa ctt aaa gcc agt cag gca agg gat ttg tta tcc	915
Ser Glu His Asn Lys Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser	
285 290 295	
aaa atg ctg gta ata gat gca tct aaa agg atc tct gta gat gaa gct	963
Lys Met Leu Val Ile Asp Ala Ser Lys Arg Ile Ser Val Asp Glu Ala	
300 305 310 315	
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Leu Gln His Pro Tyr Ile Asn Val Trp Tyr Asp Pro Ser Glu Ala Glu	
320 325 330	
gct cca cca cca aag atc cct gac aag cag tta gat gaa agg gaa cac	1059
Ala Pro Pro Pro Lys Ile Pro Asp Lys Gln Leu Asp Glu Arg Glu His	
335 340 345	
aca ata gaa gag tgg aaa gaa ttg ata tat aag gaa gtt atg gac ttg	1107
Thr Ile Glu Glu Trp Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Leu	
350 355 360	
gag gag aga acc aag aat gga gtt ata cgg ggg cag ccc tct cct tta	1155
Glu Glu Arg Thr Lys Asn Gly Val Ile Arg Gly Gln Pro Ser Pro Leu	
365 370 375	
gca cag gtg cag cag tga tcaatggctc tcagcatcca tcatcatcg	1203
Ala Gln Val Gln Gln	
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gcagtctaga agcagcagct gggcctctgg gctgctgttag atgactactt gggccatcg	1323

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Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Tyr Asp Ala Ile Leu			
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Glu Arg Asn Val Ala Ile Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln			
50	55	60	
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Met Lys Cys Val			
65	70	75	80
Asn His Lys Asn Ile Ile Gly Leu Leu Asn Val Phe Thr Pro Gln Lys			
85	90	95	
Ser Leu Glu Glu Phe Gln Asp Val Tyr Ile Val Met Glu Leu Met Asp			
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Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu Asp His Glu Arg Met			
115	120	125	
Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser			
130	135	140	
Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys			
145	150	155	160
Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala			
165	170	175	
Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg			
180	185	190	
Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Leu			
195	200	205	
Trp Ser Val Gly Cys Ile Met Gly Glu Met Val Cys His Lys Ile Leu			
210	215	220	
Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn Lys Val Ile Glu Gln			
225	230	235	240
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245	250	255	
Arg Thr Tyr Val Glu Asn Arg Pro Lys Tyr Ala Gly Tyr Ser Phe Glu			
260	265	270	
Lys Leu Phe Pro Asp Val Leu Phe Pro Ala Asp Ser Glu His Asn Lys			

275	280	285
Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile		
290	295	300
Asp Ala Ser Lys Arg Ile Ser Val Asp Glu Ala Leu Gln His Pro Tyr		
305	310	315
Ile Asn Val Trp Tyr Asp Pro Ser Glu Ala Glu Ala Pro Pro Pro Lys		
325	330	335
Ile Pro Asp Lys Gln Leu Asp Glu Arg Glu His Thr Ile Glu Glu Trp		
340	345	350
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gggcgggcga gggatctgaa acttgccac cttcgggat attgcaggac gctgcac		58
atg agc gac agt aaa tgt gac agt cag ttt tat agt gtg caa gtg gca		106
Met Ser Asp Ser Lys Cys Asp Ser Gln Phe Tyr Ser Val Gln Val Ala		
1	5	10
15		
gac tca acc ttc act gtc cta aaa cgt tac cag cag ctg aaa cca att		154
Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Gln Leu Lys Pro Ile		
20	25	30
ggc tct ggg gcc caa ggg att gtt tgt gct gca ttt gat aca gtt ctt		202
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Phe Asp Thr Val Leu		
35	40	45
ggg ata agt gtt gca gtc aag aaa cta agc cgt cct ttt cag aac caa		250
Gly Ile Ser Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln		
50	55	60
act cat gca aag aga gct tat cgt gaa ctt gtc ctc tta aaa tgt gtc		298
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Leu Lys Cys Val		
65	70	75
80		
aat cat aaa aat ata att agt ttg tta aat gtg ttt aca cca caa aaa		346
Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys		
85	90	95
act cta gaa gaa ttt caa gat gtg tat ttg gtt atg gaa tta atg gat		394
Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp		

100	105	110	
gct aac tta tgt cag gtt att cac atg gag ctg gat cat gaa aga atg Ala Asn Leu Cys Gln Val Ile His Met Glu Leu Asp His Glu Arg Met 115	120	125	442
tcc tac ctt tac cag atg ctt tgt ggt att aaa cat ctg cat tca Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser 130	135	140	490
gct ggt ata att cat aga gat ttg aag cct agc aac att gtt gtg aaa Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys 145	150	155	538
tca gac tgc acc ctg aag atc ctt gac ttt ggc ctg gcc cg aca gcg Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala 165	170	175	586
tgc act aac ttc atg atg acc cct tac gtg gtg aca cg tac tac cg Cys Thr Asn Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg 180	185	190	634
gcg ccc gaa gtc atc ctg ggt atg ggc tac aaa gag aac gtt gat atc Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile 195	200	205	682
tgg tca gtg ggt tgc atc atg gga gag ctg gtg aaa ggt tgt gtg ata Trp Ser Val Gly Cys Ile Met Gly Glu Leu Val Lys Gly Cys Val Ile 210	215	220	730
ttc caa ggc act gac cat att gat cag tgg aat aaa gtt att gag cag Phe Gln Gly Thr Asp His Ile Asp Gln Trp Asn Lys Val Ile Glu Gln 225	230	235	778
ctg gga aca cca tca gca gag ttc atg aag aaa ctt cag cca act gtg Leu Gly Thr Pro Ser Ala Glu Phe Met Lys Lys Leu Gln Pro Thr Val 245	250	255	826
agg aat tat gtc gaa aac aga cca aag tat cct gga atc aaa ttt gaa Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Pro Gly Ile Lys Phe Glu 260	265	270	874
gaa ctc ttt cca gat tgg ata ttc cca tca gaa tct gag cga gac aaa Glu Leu Phe Pro Asp Trp Ile Phe Pro Ser Glu Ser Glu Arg Asp Lys 275	280	285	922
ata aaa aca agt caa gcc aga gat ctg tta tca aaa atg tta gtg att Ile Lys Thr Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile 290	295	300	970
gat cct gac aag cg atc tct gta gac gaa gct ctg cgt cac cca tac Asp Pro Asp Lys Arg Ile Ser Val Asp Glu Ala Leu Arg His Pro Tyr 305	310	315	1018
atc act gtt tgg tat gac ccc gcc gaa gca gaa gcc cca cca cct caa Ile Thr Val Trp Tyr Asp Pro Ala Glu Ala Glu Ala Pro Pro Pro Gln 325	330	335	1066
att tat gat gcc cag ttg gaa gaa aga gaa cat gca att gaa gaa tgg Ile Tyr Asp Ala Gln Leu Glu Glu Arg Glu His Ala Ile Glu Glu Trp 340	345	350	1114
aaa gag cta att tac aaa gaa gtc atg gat tgg gaa gaa aga agc aag			1162

Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Trp Glu Glu Arg Ser Lys			
355	360	365	
aat ggt gtt gta aaa gat cag cct tca gat gca gca gta agt agc aac		1210	
Asn Gly Val Val Lys Asp Gln Pro Ser Asp Ala Ala Val Ser Ser Asn			
370	375	380	
gcc act cct tct cag tct tca tcg atc aat gac att tca tcc atg tcc		1258	
Ala Thr Pro Ser Gln Ser Ser Ile Asn Asp Ile Ser Ser Met Ser			
385	390	395	400
act gag cag acg ctg gcc tca gac aca gac agc agt ctt gat gcc tcg		1306	
Thr Glu Gln Thr Leu Ala Ser Asp Thr Asp Ser Ser Leu Asp Ala Ser			
405	410	415	
acg gga ccc ctt gaa ggc tgt cga tga taggttagaa atagcaaacc		1353	
Thr Gly Pro Leu Glu Gly Cys Arg			
420	425		
tgtcagcatt gaaggaactc tcaccctccgt gggcctgaaa tgcttggag ttgatggaac		1413	
caaataaaaa aactccatgt tctgcatgta agaaacacaa tgccctgccc tattcagacc		1473	
tgataggatt gcctgcttag atgataaaat gaggcagaat atgtctgaag aaaaaaattg		1533	
caagccacac ttcttagagat tttgttcaag atcatttcag gtgagcagtt agagtaggtg		1593	
aatttgttcaaaattgtact agtgacagtt tctcatcatc tgtaactgtt gagatgtatg		1653	
tgcattgtgac cacaaatgct tgcttggact tgcccatcta gcactttgga aatcagtatt		1713	
taaatgccaataatcttcc aggttagtgct gcttctgaag ttatctctta atcctcttaa		1773	
gtatattgg		1782	

<210> 17  
<211> 424  
<212> PRT  
<213> Homo sapiens

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1	5	10	15
Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Gln Leu Lys Pro Ile			
20	25	30	
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Phe Asp Thr Val Leu			
35	40	45	
Gly Ile Ser Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln			
50	55	60	
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Leu Lys Cys Val			
65	70	75	80
Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys			
85	90	95	
Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp			
100	105	110	

Ala Asn Leu Cys Gln Val Ile His Met Glu Leu Asp His Glu Arg Met  
 115 120 125  
 Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser  
 130 135 140  
 Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys  
 145 150 155 160  
 Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala  
 165 170 175  
 Cys Thr Asn Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg  
 180 185 190  
 Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile  
 195 200 205  
 Trp Ser Val Gly Cys Ile Met Gly Glu Leu Val Lys Gly Cys Val Ile  
 210 215 220  
 Phe Gln Gly Thr Asp His Ile Asp Gln Trp Asn Lys Val Ile Glu Gln  
 225 230 235 240  
 Leu Gly Thr Pro Ser Ala Glu Phe Met Lys Lys Leu Gln Pro Thr Val  
 245 250 255  
 Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Pro Gly Ile Lys Phe Glu  
 260 265 270  
 Glu Leu Phe Pro Asp Trp Ile Phe Pro Ser Glu Ser Glu Arg Asp Lys  
 275 280 285  
 Ile Lys Thr Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile  
 290 295 300  
 Asp Pro Asp Lys Arg Ile Ser Val Asp Glu Ala Leu Arg His Pro Tyr  
 305 310 315 320  
 Ile Thr Val Trp Tyr Asp Pro Ala Glu Ala Glu Ala Pro Pro Pro Gln  
 325 330 335  
 Ile Tyr Asp Ala Gln Leu Glu Glu Arg Glu His Ala Ile Glu Glu Trp  
 340 345 350  
 Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Trp Glu Glu Arg Ser Lys  
 355 360 365  
 Asn Gly Val Val Lys Asp Gln Pro Ser Asp Ala Ala Val Ser Ser Asn  
 370 375 380  
 Ala Thr Pro Ser Gln Ser Ser Ser Ile Asn Asp Ile Ser Ser Met Ser  
 385 390 395 400  
 Thr Glu Gln Thr Leu Ala Ser Asp Thr Asp Ser Ser Leu Asp Ala Ser  
 405 410 415  
 Thr Gly Pro Leu Glu Gly Cys Arg  
 420

<210> 18  
 <211> 3834

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (117)..(1949)

&lt;300&gt;

&lt;308&gt; M24736/Genbank

&lt;309&gt; 1994-11-07

&lt;400&gt; 18

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ccaaaaacgga aagtatttca agcctaaacc tttgggtgaa aagaactctt gaagtc atg 119

Met

1

att gct tca cag ttt ctc tca gct ctc act ttg gtg ctt ctc att aaa 167  
Ile Ala Ser Gln Phe Leu Ser Ala Leu Thr Leu Val Leu Ile Lys  
5 10 15gag agt gga gcc tgg tct tac aac acc tcc acg gaa gct atg act tat 215  
Glu Ser Gly Ala Trp Ser Tyr Asn Thr Ser Thr Glu Ala Met Thr Tyr  
20 25 30gat gag gcc agt gct tat tgt cag caa agg tac aca cac ctg gtt gca 263  
Asp Glu Ala Ser Ala Tyr Cys Gln Gln Arg Tyr Thr His Leu Val Ala  
35 40 45att caa aac aaa gaa gag att gag tac cta aac tcc ata ttg agc tat 311  
Ile Gln Asn Lys Glu Glu Ile Glu Tyr Leu Asn Ser Ile Leu Ser Tyr  
50 55 60 65tca cca agt tat tac tgg att gga atc aga aaa gtc aac aat gtg tgg 359  
Ser Pro Ser Tyr Tyr Trp Ile Gly Ile Arg Lys Val Asn Asn Val Trp  
70 75 80gtc tgg gta gga acc cag aaa cct ctg aca gaa gaa gcc aag aac tgg 407  
Val Trp Val Gly Thr Gln Lys Pro Leu Thr Glu Glu Ala Lys Asn Trp  
85 90 95gct cca ggt gaa ccc aac aat agg caa aaa gat gag gac tgc gtg gag 455  
Ala Pro Gly Glu Pro Asn Asn Arg Gln Lys Asp Glu Asp Cys Val Glu  
100 105 110atc tac atc aag aga gaa aaa gat gtg ggc atg tgg aat gat gag agg 503  
Ile Tyr Ile Lys Arg Glu Lys Asp Val Gly Met Trp Asn Asp Glu Arg  
115 120 125tgc agc aag aag aag ctt gcc cta tgc tac aca gct gcc tgt acc aat 551  
Cys Ser Lys Lys Leu Ala Leu Cys Tyr Thr Ala Ala Cys Thr Asn  
130 135 140 145aca tcc tgc agt ggc cac ggt gaa tgt gta gag acc atc aat aat tac 599  
Thr Ser Cys Ser Gly His Gly Glu Cys Val Glu Thr Ile Asn Asn Tyr  
150 155 160act tgc aag tgt gac cct ggc ttc agt gga ctc aag tgt gag caa att 647  
Thr Cys Lys Cys Asp Pro Gly Phe Ser Gly Leu Lys Cys Glu Gln Ile  
165 170 175

gtg aac tgt aca gcc ctg gaa tcc cct gag cat gga agc ctg gtt tgc 695

Val Asn Cys Thr Ala Leu Glu Ser Pro Glu His Gly Ser Leu Val Cys			
180	185	190	
agt cac cca ctg gga aac ttc agc tac aat tct tcc tgc tct atc agc			743
Ser His Pro Leu Gly Asn Phe Ser Tyr Asn Ser Ser Cys Ser Ile Ser			
195	200	205	
tgt gat agg ggt tac ctg cca agc agc atg gag acc atg cag tgt atg			791
Cys Asp Arg Gly Tyr Leu Pro Ser Ser Met Glu Thr Met Gln Cys Met			
210	215	220	225
tcc tct gga gaa tgg agt gct att cca gcc tgc aat gtg gtt gag			839
Ser Ser Gly Glu Trp Ser Ala Pro Ile Pro Ala Cys Asn Val Val Glu			
230	235	240	
tgt gat gct gtg aca aat cca gcc aat ggg ttc gtg gaa tgt ttc caa			887
Cys Asp Ala Val Thr Asn Pro Ala Asn Gly Phe Val Glu Cys Phe Gln			
245	250	255	
aac cct gga agc ttc cca tgg aac aca acc tgt aca ttt gac tgt gaa			935
Asn Pro Gly Ser Phe Pro Trp Asn Thr Cys Thr Phe Asp Cys Glu			
260	265	270	
gaa gga ttt gaa cta atg gga gcc cag agc ctt cag tgt acc tca tct			983
Glu Gly Phe Glu Leu Met Gly Ala Gln Ser Leu Gln Cys Thr Ser Ser			
275	280	285	
ggg aat tgg gac aac gag aag cca acg tgt aaa gct gtg aca tgc agg			1031
Gly Asn Trp Asp Asn Glu Lys Pro Thr Cys Lys Ala Val Thr Cys Arg			
290	295	300	305
gcc gtc cgc cag cct cag aat ggc tct gtg agg tgc agc cat tcc cct			1079
Ala Val Arg Gln Pro Gln Asn Gly Ser Val Arg Cys Ser His Ser Pro			
310	315	320	
gct gga gag ttc acc ttc aaa tca tcc tgc aac ttc acc tgt gag gaa			1127
Ala Gly Glu Phe Thr Phe Lys Ser Ser Cys Asn Phe Thr Cys Glu Glu			
325	330	335	
ggc ttc atg ttg cag gga cca gcc cag gtt gaa tgc acc act caa ggg			1175
Gly Phe Met Leu Gln Gly Pro Ala Gln Val Glu Cys Thr Thr Gln Gly			
340	345	350	
cag tgg aca cag caa atc cca gtt tgt gaa gct ttc cag tgc aca gcc			1223
Gln Trp Thr Gln Gln Ile Pro Val Cys Glu Ala Phe Gln Cys Thr Ala			
355	360	365	
ttg tcc aac ccc gag cga ggc tac atg aat tgt ctt cct agt gct tct			1271
Leu Ser Asn Pro Glu Arg Gly Tyr Met Asn Cys Leu Pro Ser Ala Ser			
370	375	380	385
ggc agt ttc cgt tat ggg tcc agc tgt gag ttc tcc tgt gag cag ggt			1319
Gly Ser Phe Arg Tyr Gly Ser Ser Cys Glu Phe Ser Cys Glu Gln Gly			
390	395	400	
ttt gtg ttg aag gga tcc aaa agg ctc caa tgt ggc ccc aca ggg gag			1367
Phe Val Leu Lys Gly Ser Lys Arg Leu Gln Cys Gly Pro Thr Gly Glu			
405	410	415	
tgg gac aac gag aag ccc aca tgt gaa gct gtg aga tgc gat gct gtc			1415
Trp Asp Asn Glu Lys Pro Thr Cys Glu Ala Val Arg Cys Asp Ala Val			
420	425	430	

cac cag ccc ccg aag ggt ttg gtg agg tgt gct cat tcc cct att gga	1463
His Gln Pro Pro Lys Gly Leu Val Arg Cys Ala His Ser Pro Ile Gly	
435 440 445	
gaa ttc acc tac aag tcc tct tgt gcc ttc agc tgt gag gag gga ttt	1511
Glu Phe Thr Tyr Lys Ser Ser Cys Ala Phe Ser Cys Glu Glu Gly Phe	
450 455 460 465	
gaa tta tat gga tca actcaa ctt gag tgc aca tct cag gga caa tgg	1559
Glu Leu Tyr Gly Ser Thr Gln Leu Glu Cys Thr Ser Gln Gly Gln Trp	
470 475 480	
aca gaa gag gtt cct tcc tgc caa gtg gta aaa tgt tca agc ctg gca	1607
Thr Glu Glu Val Pro Ser Cys Gln Val Val Lys Cys Ser Ser Leu Ala	
485 490 495	
gtt ccg gga aag atc aac atg agc tgc agt ggg gag ccc gtg ttt ggc	1655
Val Pro Gly Lys Ile Asn Met Ser Cys Ser Gly Glu Pro Val Phe Gly	
500 505 510	
act gtg tgc aag ttc gcc tgt cct gaa gga tgg acg ctc aat ggc tct	1703
Thr Val Cys Lys Phe Ala Cys Pro Glu Gly Trp Thr Leu Asn Gly Ser	
515 520 525	
gca gct cg <sup>g</sup> aca tgt gga gcc aca gga cac tgg tct ggc ctg cta cct	1751
Ala Ala Arg Thr Cys Gly Ala Thr Gly His Trp Ser Gly Leu Leu Pro	
530 535 540 545	
acc tgt gaa gct ccc act gag tcc aac att ccc t <sup>t</sup> g gta gct gga ctt	1799
Thr Cys Glu Ala Pro Thr Glu Ser Asn Ile Pro Leu Val Ala Gly Leu	
550 555 560	
tct gct gct gga ctc tcc ctc ctg aca tta gca cca ttt ctc ctc tgg	1847
Ser Ala Ala Gly Leu Ser Leu Leu Thr Leu Ala Pro Phe Leu Leu Trp	
565 570 575	
ctt cgg aaa tgc tta cgg aaa gca aag aaa ttt gtt cct gcc agc agc	1895
Leu Arg Lys Cys Leu Arg Lys Ala Lys Lys Phe Val Pro Ala Ser Ser	
580 585 590	
tgc caa agc ctt gaa tca gac gga agc tac caa aag cct tct tac atc	1943
Cys Gln Ser Leu Glu Ser Asp Gly Ser Tyr Gln Lys Pro Ser Tyr Ile	
595 600 605	
ctt taa gttcaaaaaga atcagaaaaca ggtgcatactg ggaaactaga gggatacact	1999
Leu	
610	
gaagttaaca gagacagata actctccctcg ggtctctggc ccttcttgcc tactatgcc	2059
gatgccttta tggctgaaac cgcaacaccc atcaccactt caatagatca aagtccagca	2119
ggcaaggacg gccttcaact gaaaagactc agtgtccct ttcctactct caggatcaag	2179
aaagtgttgg ctaatgaagg gaaaggatat tttcttccaa gcaaaggtaa agagaccaag	2239
actctgaaat ctcagaattc ctttcttaac tctcccttgc tcgctgtaaa atcttggcac	2299
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ctaaaaatataacttaa aaaaatgaca gatgttgaat gcccacagggc aaatgcata	2479

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 taatcacttt catccctatg ggattcagtg cttcttaaag agttcttaag gattgtgata 2599  
 ttttacttg cattgaatat attataatct tccatacttc ttcattcaat acaagtgtgg 2659  
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 agattacccc ctcattgttt attaacaat tatgttacat ctgtttaaa ttatttcaa 2779  
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 atgaatgtct ttgttggaaa agaatacaga aagatggatg tgctttgcat tcctacaaag 3079  
 atgttgtca gatgtgatata gtaaacataa ttcttgata ttatgaaaga tttaaatttc 3139  
 acaatagaaa ctcaccatgt aaaagagtca tctggtagat tttaacgaa tgaagatgtc 3199  
 taatagttat tccctatttg tttcttctg tatgttaggg tgctctggaa gagaggaatg 3259  
 cctgtgtgag caagcattta tgtttattta taagcagatt taacaattcc aaaggaatct 3319  
 ccagtttca gttgatcact ggcaatgaaa aattctcagt cagtaattgc caaagctgct 3379  
 ctagccttga ggagtgtgag aatcaaaact ctcctacact tccattaact tagcatgtgt 3439  
 tgaaaaaaaaa agttcagag aagttctggc tgaacactgg caacgacaaa gccaacagtc 3499  
 aaaacagaga tgtgataagg atcagaacag cagaggttct tttaaagggg cagaaaaact 3559  
 ctggaaata agagagaaca actactgtga tcaggctatg tatgaaatac agtgttattt 3619  
 tctttgaaat tgtttaagtg ttgttaaatat ttatgtaaac tgcattagaa attagctgtg 3679  
 tgaaatacca gtgtggttt tgtttagtt ttattgagaa tttaaattta taactaaaa 3739  
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 cactataaag gttgacaata aatgtgctta tgttt 3834

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 <211> 610  
 <212> PRT  
 <213> Homo sapiens

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 Met Ile Ala Ser Gln Phe Leu Ser Ala Leu Thr Leu Val Leu Leu Ile  
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Lys Glu Ser Gly Ala Trp Ser Tyr Asn Thr Ser Thr Glu Ala Met Thr  
 20 25 30

Tyr Asp Glu Ala Ser Ala Tyr Cys Gln Gln Arg Tyr Thr His Leu Val  
 35 40 45

Ala Ile Gln Asn Lys Glu Glu Ile Glu Tyr Leu Asn Ser Ile Leu Ser

50	55	60
Tyr Ser Pro Ser Tyr Tyr Trp Ile Gly Ile Arg Lys Val Asn Asn Val		
65	70	75
80		
Trp Val Trp Val Gly Thr Gln Lys Pro Leu Thr Glu Glu Ala Lys Asn		
85	90	95
Trp Ala Pro Gly Glu Pro Asn Asn Arg Gln Lys Asp Glu Asp Cys Val		
100	105	110
Glu Ile Tyr Ile Lys Arg Glu Lys Asp Val Gly Met Trp Asn Asp Glu		
115	120	125
Arg Cys Ser Lys Lys Lys Leu Ala Leu Cys Tyr Thr Ala Ala Cys Thr		
130	135	140
Asn Thr Ser Cys Ser Gly His Gly Glu Cys Val Glu Thr Ile Asn Asn		
145	150	155
160		
Tyr Thr Cys Lys Cys Asp Pro Gly Phe Ser Gly Leu Lys Cys Glu Gln		
165	170	175
Ile Val Asn Cys Thr Ala Leu Glu Ser Pro Glu His Gly Ser Leu Val		
180	185	190
Cys Ser His Pro Leu Gly Asn Phe Ser Tyr Asn Ser Ser Cys Ser Ile		
195	200	205
Ser Cys Asp Arg Gly Tyr Leu Pro Ser Ser Met Glu Thr Met Gln Cys		
210	215	220
Met Ser Ser Gly Glu Trp Ser Ala Pro Ile Pro Ala Cys Asn Val Val		
225	230	235
240		
Glu Cys Asp Ala Val Thr Asn Pro Ala Asn Gly Phe Val Glu Cys Phe		
245	250	255
Gln Asn Pro Gly Ser Phe Pro Trp Asn Thr Thr Cys Thr Phe Asp Cys		
260	265	270
Glu Glu Gly Phe Glu Leu Met Gly Ala Gln Ser Leu Gln Cys Thr Ser		
275	280	285
Ser Gly Asn Trp Asp Asn Glu Lys Pro Thr Cys Lys Ala Val Thr Cys		
290	295	300
Arg Ala Val Arg Gln Pro Gln Asn Gly Ser Val Arg Cys Ser His Ser		
305	310	315
320		
Pro Ala Gly Glu Phe Thr Phe Lys Ser Ser Cys Asn Phe Thr Cys Glu		
325	330	335
Glu Gly Phe Met Leu Gln Gly Pro Ala Gln Val Glu Cys Thr Thr Gln		
340	345	350
Gly Gln Trp Thr Gln Gln Ile Pro Val Cys Glu Ala Phe Gln Cys Thr		
355	360	365
Ala Leu Ser Asn Pro Glu Arg Gly Tyr Met Asn Cys Leu Pro Ser Ala		
370	375	380
Ser Gly Ser Phe Arg Tyr Gly Ser Ser Cys Glu Phe Ser Cys Glu Gln		

385	390	395	400
Gly Phe Val Leu Lys	Gly Ser Lys Arg	Leu Gln Cys Gly Pro Thr Gly	
405		410	415
Glu Trp Asp Asn Glu Lys Pro Thr Cys	Glu Ala Val Arg Cys Asp Ala		
420	425		430
Val His Gln Pro Pro Lys Gly	Leu Val Arg Cys Ala His Ser Pro Ile		
435	440		445
Gly Glu Phe Thr Tyr Lys Ser	Ser Cys Ala Phe Ser Cys Glu Glu Gly		
450	455	460	
Phe Glu Leu Tyr Gly Ser Thr Gln	Leu Glu Cys Thr Ser Gln Gly Gln		
465	470	475	480
Trp Thr Glu Glu Val Pro Ser Cys Gln	Val Val Lys Cys Ser Ser Leu		
485	490		495
Ala Val Pro Gly Lys Ile Asn Met	Ser Cys Ser Gly Glu Pro Val Phe		
500	505	510	
Gly Thr Val Cys Lys Phe Ala Cys Pro Glu Gly Trp	Thr Leu Asn Gly		
515	520	525	
Ser Ala Ala Arg Thr Cys Gly	Ala Thr Gly His Trp Ser Gly Leu Leu		
530	535	540	
Pro Thr Cys Glu Ala Pro Thr Glu Ser Asn	Ile Pro Leu Val Ala Gly		
545	550	555	560
Leu Ser Ala Ala Gly	Leu Ser Leu Leu Thr Leu Ala Pro Phe Leu Leu		
565	570	575	
Trp Leu Arg Lys Cys Leu Arg Lys	Ala Lys Lys Phe Val Pro Ala Ser		
580	585	590	
Ser Cys Gln Ser Leu Glu Ser Asp	Gly Ser Tyr Gln Lys Pro Ser Tyr		
595	600	605	
Ile Leu			
610			

<210> 20  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> PCR primer

<400> 20  
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25

<210> 21  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>

<223> PCR primer

<400> 21

ttctgattct tttgaactta aaggat

26

<210> 22

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 22

cgcggatccg cgtactcaga gtt

23

<210> 23

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 23

cggaattccg ttcagggagg cgt

23

<210> 24

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 24

cttaaaatgc ctgggaagat ggtcgt

26

<210> 25

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 25

atcaaggatt agtacactt ttgatt

26

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/28965

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : CO7H 21/04, 21/02; C12Q 1/68; A61K 48/00  
 US CL : 536/ 23.1, 24.3, 24.31, 24.33, 24.5; 435/6, 91.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/ 23.1, 24.3, 24.31, 24.33, 24.5; 435/6, 91.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,656,612 A (MONIA et al) 12 August 1997 (12.08.1997), column 17, line 19.	26-33
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Y		14-18
X	WO 92-22651 A1 (ISIS PHARMACEUTICALS, INC.) 23 December 1992 (23.12.92), page 15, lines 1-8.	26-33
---		-----
Y		14-18
X,P	US 5,877,309 A (MCKAY et al) 02 March 1999 (02.03.99), col. 43.	34-38
---		-----
Y,P		1-3, 9-13, 26-28
A	MIN W. et al TNF Initiates E-selectin Transcription in Human Endothelial Cell Through Parallel TRAF-NF-kB and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 Pathways Journal of Immunology. July 1997, Vol. 159. No. 7, pages 3508-3518, especially page 3515.	1-3, 9-13, 26-28, 34-38
X	US 5,405,941 A (JOHNSON) 11 April 1995 (11.04.95), column 12, lines 18-34.	14-15

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search

15 February 2000 (15.02.2000)

Date of mailing of the international search report

08 MAR 2000

Name and mailing address of the ISA/US  
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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US99/28965

**Continuation of B. FIELDS SEARCHED Item 3:** USPAT, EPO, JPO, Derwent, Caplus, Registry  
search terms: antisense, aptamer, triplex, ribozyme, oligonucleotide, TNF-a, "tumor necrosis factor", cell adhesion, ICAM-1, E-selectin, VCAM-1.